Docket No. 41426-GC/JPW/MAF

Application
for
United States Letters Patent

To all whom it may concern:

Be it known that

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have invented certain new and useful improvements in

PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

of which the following is a full, clear and exact description.

PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

This invention disclosed herein was made in part with Government support under Grants Nos. DK47650 and CA58192, CA-39203, CA-29502, CA-08748-29 from the National Institute of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Throughout this application various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of each set of Examples in the Experimental Details section.

Prostate cancer is among the most significant medical problems in the United States, as the disease is now the most common malignancy diagnosed in American males. In 1992 there were over 132,000 new cases of prostate cancer detected with over 36,000 deaths attributable to the disease, representing a 17.3% increase over 4 years (8). Five year survival rates for patients with prostate cancer range from 88% for those with localized disease to 29% for those with metastatic disease. The

rapid increase in the number of cases appears to result in part from an increase in disease awareness as well as the widespread use of clinical markers such as the secreted proteins prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP) (7).

The prostate gland is a site of significant pathology affected by conditions such as benign growth (BPH), neoplasia (prostatic cancer) and infection (prostatitis). Prostate cancer represents the second leading cause of death from cancer in man (7). However prostatic cancer is the leading site for cancer development in men. The difference between these two facts relates to prostatic cancer occurring with increasing frequency as men age, especially in the ages beyond 60 at a time when death from other factors often intervenes. Also, the spectrum of biologic aggressiveness of prostatic cancer is great, so that in some men following detection the tumor remains a latent histologic tumor and does not become clinically significant, whereas in other it progresses rapidly, metastasizes and kills the man in a relatively short 2-5 year period (7 and 59).

In prostate cancer cells, two specific proteins that are made in very high concentrations are prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) (21, 47, and 65). These proteins have been characterized and have been used to follow response to therapy. With the development of cancer, the normal architecture of the gland becomes altered, including loss of the normal duct structure for the removal of secretions and thus the secretions reach the serum. Indeed measurement of serum PSA is suggested as a potential screening method for prostatic cancer. Indeed, the relative amount of PSA and/or PAP in the cancer reduces as compared to normal or benign tissue.

PAP was one of the earliest serum markers for detecting metastatic spread (47). PAP hydrolyses tyrosine phosphate and has a broad substrate specificity. Tyrosine phosphorylation is often increased with oncogenic transformation. It has been hypothesized that during neoplastic transformation there is less phosphatase activity available to inactivate proteins that are activated by phosphorylation on tyrosine residues. In some instances, insertion of phosphatases that have tyrosine phosphatase activity has reversed the malignant phenotype.

PSA is a protease and it is not readily appreciated how loss of its activity correlates with cancer development (21, and 65). The proteolytic activity of PSA is inhibited by zinc. Zinc concentrations are high in the normal prostate and reduced in prostatic cancer. Possibly the loss of zinc allows for increased proteolytic activity by PSA. As proteases are involved in metastasis and some proteases stimulate mitotic activity, the potentially increased activity of PSA could be hypothesized to play a role in the tumors metastases and spread (39).

Both PSA and PAP are found in prostatic secretions. Both appear to be dependent on the presence of androgens for their production and are substantially reduced following androgen deprivation.

Prostate-specific membrane antigen (PSM) which appears to be localized to the prostatic membrane has been identified. This antigen was identified as the result of generating monoclonal antibodies to a prostatic cancer cell, LNCaP (22).

Dr. Horoszewicz established a cell line designated LNCaP from the lymph node of a hormone refractory,

heavily pretreated patient (23). This line was found to have an aneuploid human male karyotype. It maintained prostatic differentiation functionality in that it produced both PSA and PAP. It possessed an androgen receptor of high affinity and specificity. Mice were immunized with LNCaP cells and hybridomas were derived from sensitized animals. A monoclonal antibody was derived and was designated 7E11-C5 (22). The antibody staining was consistent with a membrane location and isolated fractions of LNCaP cell membranes exhibited a strongly positive reaction with immunoblotting and ELISA techniques. This antibody did not inhibit or enhance the growth of LNCaP cells in vitro or in vivo. The antibody to this antigen was remarkably specific to prostatic epithelial cells, as no reactivity was observed in any other component. Immunohistochemical staining of cancerous epithelial cells was more intense than that of normal or benign epithelial cells.

Dr. Horoszewicz also reported detection of immunoreactive material using 7E11-C5 in serum of prostatic cancer patients (22). The immunoreactivity was detectable in nearly 60% of patients with stage D-2 disease and in a slightly lower percentage of patients with earlier stage disease, but the numbers of patients in the latter group are small. Patients with benign prostatic hyperplasia (BPH) were negative. Patients with no apparent disease were negative, but 50-60% of patients in remission yet with active stable disease or with progression demonstrated positive serum reactivity. Patients with non prostatic tumors did not show immunoreactivity with 7E11-C5.

The 7E11-C5 monoclonal antibody is currently in clinical trials. The aldehyde groups of the antibody were oxidized and the linker-chelator glycol-tyrosyl-

(n, ε-diethylenetriamine-pentacetic acid)-lysine (GYK-DTPA) was coupled to the reactive aldehydes of the heavy chain. The resulting antibody was designated CYT-356. Immunohistochemical staining patterns were similar except that the CYT-356 modified antibody stained skeletal muscle. The comparison of CYT-356 with 7E11-C5 monoclonal antibody suggested both had binding to type 2 muscle fibers. The reason for the discrepancy with the earlier study, which reported skeletal muscle to be negative, was suggested to be due to differences in tissue fixation techniques. Still, the most intense and definite reaction was observed with prostatic epithelial cells, especially cancerous cells. Reactivity with mouse skeletal muscle was detected with immunohistochemistry but not in imaging studies. The $Indium^{111}$-labeled antibody localized to LNCaP tumors grown in nude mice with an uptake of nearly 30% of the injected dose per gram tumor at four days. In-vivo, no selective retention of the antibody was observed in antigen negative tumors such as PC-3 and DU-145, or by skeletal muscle. Very little was known about the PSM antigen. An effort at purification and characterization has been described at meetings by Dr. George Wright and colleagues (14 and 64).

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1C: Immunohistochemical detection of PSM antigen expression in prostate cell lines. Top panel reveals uniformly high level of expression in LNCaP cells; middle panel and lower panel are DU-145 and PC-3 cells respectively, both negative.

Figure 2: Autoradiogram of protein gel revealing products of PSM coupled *in-vitro* transcription/translation. Non-glycosylated PSM polypeptide is seen at 84 kDa (lane 1) and PSM glycoprotein synthesized following the addition of microsomes is seen at 100 kDa (lane 2).

Figure 3: Western Blot analysis detecting PSM expression in transfected non-PSM expressing PC-3 cells. 100 kDa PSM glycoprotein species is clearly seen in LNCaP membranes (lane 1), LNCaP crude lysate (lane 2), and PSM-transfected PC-3 cells (lane 4), but is undetectable in native PC-3 cells (lane 3).

Figure 4: Autoradiogram of ribonuclease protection gel assaying for PSM mRNA expression in normal human tissues. Radiolabeled 1 kb DNA ladder (Gibco-BRL) is shown in lane 1. Undigested probe is 400 nucleotides (lane 2), expected protected PSM band is 350 nucleotides, and tRNA control is shown

(lane 3). A strong signal is seen in human prostate (lane 11), with very faint, but detectable signals seen in human brain (lane 4) and human salivary gland (lane 12). No signal was detected in lane 5 kidney, lane 6 liver, lane 7 lung, lane 8 mammary gland, lane 9 pancreas, lane 10 placenta, lane 13 skeletal muscle, lane 14 spleen, and lane 15 testes.

Figure 5: Autoradiogram of ribonuclease protection gel assaying for PSM mRNA expression in LNCaP tumors grown in nude mice, and in human prostatic tissues. ^{32}P-labeled 1 kb DNA ladder is shown in lane 1. 298 nucleotide undigested probe is shown (lane 2), and tRNA control is shown (lane 3). PSM mRNA expression is clearly detectable in LNCaP cells (lane 4), orthotopically grown LNCaP tumors in nude mice with and without matrigel (lanes 5 and 6), and subcutaneously implanted and grown LNCaP tumors in nude mice (lane 7). PSM mRNA expression is also seen in normal human prostate (lane 8), and in a moderately differentiated human prostatic adenocarcinoma (lane 10). Very faint expression is seen in a sample of human prostate tissue with benign hyperplasia (lane 9).

Figure 6: Ribonuclease protection assay for PSM expression in LNCaP cells treated with physiologic doses of various steroids for 24 hours. ^{32}P-labeled DNA ladder is

shown in lane 1. 298 nucleotide undigested probe is shown (lane 2), and tRNA control is shown (lane 3). PSM mRNA expression is highest in untreated LNCaP cells in charcoal-stripped media (lane 4). Applicant see significantly diminished PSM expression in LNCaP cells treated with DHT (lane 5), Testosterone (lane 6), Estradiol (lane 7), and Progesterone (lane 8), with little response to Dexamethasone (lane 9).

Figure 7: Data illustrating results of PSM DNA and RNA presence in transfect Dunning cell lines employing Southern and Northern blotting techniques

Figures 8A-8B:

Figure A indicates the power of cytokine transfected cells to teach unmodified cells. Administration was directed to the parental flank or prostate cells. The results indicate the microenvironment considerations.

Figure B indicates actual potency at a particular site. The tumor was implanted in prostate cells and treated with immune cells at two different sites.

Figures 9A-9B:

Relates potency of cytokines in inhibiting growth of primary tumors. Animals administered un-modified parental tumor cells and administered

as a vaccine transfected cells. Following prostatectomy of rodent tumor results in survival increase.

Figure 10: PCR amplification with nested primers improved the level of detection of prostatic cells from approximately one prostatic cell per 10,000 MCF-7 cells to better than one cell per million MCF-7 cells, using PSA.

Figure 11: PCR amplification with nested primers improved the level of detection of prostatic cells from approximately one prostatic cell per 10,000 MCF-7 cells to better than one cell per million MCF-7 cells, using PSM-derived primers.

Figure 12: A representative ethidium stained gel photograph for PSM-PCR. Samples run in lane A represent PCR products generated from the outer primers and samples in lanes labeled B are products of inner primer pairs.

Figure 13: PSM Southern blot autoradiograph. The sensitivity of the Southern blot analysis exceeded that of ethidium staining, as can be seen in several samples where the outer product is not visible, but is detectable by Southern blotting.

Figure 14: Characteristics of the 16 patients analyzed with respect to their clinical stage, treatment, serum PSA and PAP values, and results of assay.

Figures 15A-15D: DNA sequence containing promoter elements from nucleotide -1 to nucleotide -3017. -1 is upstream of start site of PSM.

Figure 16: Potential binding sites on the PSM promoter fragment.

Figure 17: Promoter activity of PSM up-stream fragment/CAT gene chimera.

Figure 18: Comparison between PSM and PSM' cDNA. Sequence of the 5' end of PSM cDNA (32) is shown. Underlined region (beginning at nucleotide 115 and continuing to nucleotide 380) denotes nucleotides which are absent in PSM' cDNA but present in PSM cDNA.. Boxed region represents the putative transmembrane domain of PSM antigen. * Asterisk denotes the putative translation initiation site for PSM'.

Figure 19: Graphical representation of PSM and PSM' cDNA sequences and antisense PSM RNA probe (b). PSM cDNA sequence with complete coding region (32). (a) PSM' cDNA sequence from this study. (c) Cross hatched and open boxes denote sequences identity in PSM and PSM'. Hatched box indicates sequence absent from PSM'. Regions of cDNA sequence complementary to the antisense probe are indicated by dashed lines between the sequences.

Figure 20: RNase protection assay with PSM specific probe in primary prostatic tissues. Total cellular RNA was isolated from human prostatic samples: normal prostate, BPH, and CaP. PSM and PSM' spliced variants are indicated with arrows at right. The left lane is a DNA ladder. Samples from different patients are classified as: lanes 3-6, *CaP*, carcinoma of prostate; *BPH*, benign prostatic hypertrophy, lanes 7-9; *normal*, normal prostatic tissue, lanes 10-12. Autoradiograph was exposed for longer period to read lanes 5 and 9.

Figure 21: Tumor Index, a quantification of the expression of PSM and PSM'. Expression of PSM and PSM' was quantified by densitometry and expressed as a ratio of PSM/PSM' on the Y-axis. Three samples each were quantitated for primary CaP, BPH and normal prostate tissues. Two samples were quantitated for LNCaP. *Normal*, normal prostate tissue.

Figure 22: Characterization of PSM membrane bound and PSM' in the cytosol.

Figure 23: Photograph of ethidium bromide stained gel depicting representative negative and positive controls used in the study. Samples 1-5 were from, respectively: male with prostatis, a healthy female volunteer, a male with BPH, a control 1:1,000,000 dilution of LNCaP cells, and a patient with renal

cell carcinoma. Below each reaction is the corresponding control reaction performed with beta-2-microglobulin primers to assure RNA integrity. No PCR products were detected for any of these negative controls.

Figure 24: Photograph of gel displaying representative positive PCR results using PSM primers in selected patients with either localized or disseminated prostate cancer. Sample 1-5 were from. respectively: a patient with clinically localized stage $T1_c$ disease, a radical prostatectomy patient with organ confined disease and a negative serum PSA, a radical prostatectomy patient with locally advanced disease and a negative serum PSA, a patient with treated stage D2 disease, and a patient with treated hormone refractory disease.

Figure 25: Chromosomal location of PSM based on in-situ hybridization with cDNA and with genomic cosmids.

Figure 26: Human monochromosomal somatic cell hybrid blot showing that chromosome 11 contained the PSM genetic sequence by Southern analysis. DNA panel digested with PstI restriction enzyme and probed with PSM cDNA. Lanes M and H refer to mouse and hamster DNAs. The numbers correspond to the human chromosomal DNA in that hybrid.

Figure 27: Ribonuclease protection assay using PSM radiolabeled RNA probe revels an abundant PSM mRNA expression in AT6.1-11 clone 1, but not in AT6.1-11 clone 2, thereby mapping PSM to 11p11.2-13 region.

Figure 28: Tissue specific expression of PSM RNA by Northern blotting and RNAse protection assay.

Figure 29: Mapping of the PSM gene to the 11p11.2-p13 region of human chromosome 11 by southern blotting and in-situ hybridization.

Figure 30: Schematic of potential response elements.

Figure 31: Schematic depiction of metastatic prostate cell transfected with promoter for PSM which is driving expression of prodrug activating enzyme cytosine deaminase. This allows for prostate specific expression and tumor localized conversion of non-toxic 5 fluorocytosine to 5 flurouracil.

Figure 32A-32C:

Nucleic acid of PSM genomic DNA is read 5 prime away from the transcription start site: number on the sequences indicates nucleotide upstream from the start site. Therefore, nucleotide #121 is actually -121 using conventional numbering system.

Figure 33: Representation of NAAG 1, acividin, azotomycin, and 6-diazo-5-oxo-norleucine, DON.

Figure 34: Representation of N-acetylaspartylglutamate (NAAG), PALA, PALAGLU, phosphonate antagonist of glutamate receptor and phosponates of PALAGLU and NAAG.

Figure 35: Synthesis of N-acetylaspartylglutamate, NAAG 1.

Figure 36: Synthesis of N-phosphonoacetylaspartyl-L-glutamate.

Figure 37: Synthesis of 5-diethylphosphonon-2 amino benzylvalerate intermediate.

Figure 38: Synthesis of analog 4 and 5.

Figure 39: Representation of DON, analogs 17-20.

Figure 40: Substrates for targeted drug delivery, analog 21 and 22.

Figure 41: Dynemycin A and its mode of action.

Figure 42: Synthesis of analog 28.

Figure 43: Synthesis for intermediate analog 28.

Figure 44: Attachment points for PALA.

Figure 45: Mode of action for substrate 21.

Figures 46A-46D:

Intron 1F: Forward Sequence.

Figures 47A-47E:

Intron 1R: Reverse Sequence

Figures 48A-48C:

Intron 2F: Forward Sequence

Figures 49A-49C:

Intron 2R: Reverse Sequence

Figures 50A-50B:

Intron 3F: Forward Sequence

Figures 51A-51B:

Intron 3R: Reverse Sequence

Figures 52A-52C:

Intron 4F: Forward Sequence

Figures 53A-53E:

Intron 4RF: Reverse Sequence

Figure 54: PSM genomic organization of the exon and 19 intron junction sequences. The exon/intron junctions are as follows:

1. Exon /intron 1 at bp 389-390;
2. Exon /intron 2 at bp 490-491;
3. Exon /intron 3 at bp 681-682;
4. Exon /intron 4 at bp 784-785;
5. Exon /intron 5 at bp 911-912;
6. Exon /intron 6 at bp 1096-1097;
7. Exon /intron 7 at bp 1190-1191;
8. Exon /intron 8 at bp 1289- 1290;
9. Exon /intron 9 at bp 1375-1376;

10. Exon /intron 10 at bp 1496-1497;
11. Exon /intron 11 at bp 1579-1580;
12. Exon /intron 12 at bp 1643-1644;
13. Exon /intron 13 at bp 1710-1711;
14. Exon /intron 14 at bp 1803-1804;
15. Exon /intron 15 at bp 1894-1895;
16. Exon /intron 16 at bp 2158-2159;
17. Exon /intron 17 at bp 2240-2241;
18. Exon /intron 18 at bp 2334-2335;
19. Exon /intron 19 at bp 2644-2645.

Figures 55A-55J: Alternatively spliced PSM (PSM')nucleic acid sequence and amino acid sequence.

Figure 56: PSM pteroyl (folate) hydrolase activity in LNCaP membrane preparation. Time course of $MTXglu_3$ hydrolysis (- ■ -) and concurrent formation of $MTXglu_2$ (- -), $MTXglu_1$ (- ▲ -), and MTX (- -), respectively. Membrane fractions were prepared as described in Methods. Reaction volume was 100 μL containing 50 mM acetate/Triton buffer pH 4.5, 50 μM $MTXglu_3$, 10 μg/mL protein. Values are $x \pm$ S.D. from three separate LNCaP membrane preparations.

Figure 57: PSM pteroyl (folate) hydrolase activity of immunoprecipitated PSM antigen. Diagram shows typical capillary electrophoretic separation patterns of $MTXglu_{(n)}$ derivatives at 0, 30, 60 and 240 minute reaction times. Elution intervals for $MTXglu_3$, $MTXglu_2$, $MTXglu_1$, and MTX are 4.25, 3.95, 3.55, and 3.06 min, respectively. Total volume of

reaction mixture was 100 uL containing 50 uM $MTXglu_3$.

Figure 58: Effects of pH on gamma-glutamyl hydrolase (PSM hydrolase) activity in LNCaP, PC-3 PSM-transfected (PC-3(+)) and PSM non-transfected (PC-3(-)) cells. Enzymic activity is reported as μM $MTXglu_2$ formed/mg protein. Each value represents the mean of 3 reactions containing 50-60 $\mu g/mL$ protein. The following buffers were used in 50 mM concentrations spanning a pH range of 2 to 10: glycine-HCl, pH 2.2 to 3.6; acetate, pH 3.6 to 5.6; 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.6 to 6.8; Tris(hydroxymethyl)aminomethane (TRIS), pH 7 to 8.5; and glycine-NaOH, pH 8.6 to 10.0.

Figure 59: Comparison of pteroyl hydrolase activity in membranes isolated from LNCaP, PC-3, TSU-Pr1, and Duke-145 adenocarcinoma cell lines. Membranes were isolated as described in Methods. Each value represents the mean of triplicate reactions normalized to 1 mg/mL protein.

Figure 60A-60C:

Immunohistochemical analysis of LNCaP and PC-3 PSM-transfected and PSM-non-transfected cells. A 2.65 kb PSM cDNA containing a hygromycin selection vector was cloned into non PSM-antigen expressing PC-3 cells and maintained in

regular media supplemented with hygromycin B. As a control, PC-3 cells were also transfected with the pREP7 vector alone (PC-3 PSM non-transfected cells). Cells were permeabilized in acetone/methanol (1:1 v/v) mixture, blocked with 5% bovine serum albumin/Tris buffered saline (TBS) and the 7E11-C5 monoclonal PSM antibody was added to cells. A secondary anti-mouse IgG_1 antibody conjugated with alkaline phosphatase was added and PSM-positive cell staining performed with bromochloroindolylphenol phosphate. Panel A demonstrates intense immunoreactivity associated with LNCaP cells using the monoclonal PSM antibody; In panel B, comparable staining occurs in PC-3 cells transfected with PSM expression vector. Panel C illustrates PC-3 cells expressing pREP7 hygromycin vector alone.

Figure 61: Comparison of pteroyl (folate) hydrolase activity in membranes isolated from PSM expressing PC-3 cells and PC-3 cells expressing pREP7 hygromycin vector alone. Membranes were isolated as described in Methods. Each value represents the mean of triplicate reactions normalized to 1 mg/mL protein.

Figure 62: Representation of N-acetylaspartylglutamate (NAAG), folic acid, folate-gamma-polyglutamate,

methotrexate, methotrexate-gamma-polyglutamate, methotrexate-alpha-monoglutamate, methotrexate-gamma-diglutamate, methotrexate-gamma-triglutamate, methotrexate-gamma-tetraglutamate.

Figure 63A-63B: Solid phase synthesis of methotrexate alpha-polyglutamatae analogs.

Figure 64: Sequence analysis of microsatellite instability in PSM gene.

Figure 65: PSM genomic organization.

Figure 66: Location of microsatellite in PSM gene

SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid molecule encoding an alternatively spliced human prostate-specific membrane antigen. This invention provides an isolated nucleic acid comprising a promoter sequence normally associated with the transcription of a gene encoding a human prostate-specific membrane antigen. This invention provides an isolated polypeptide having the biological activity of an alternatively spliced prostate-specific membrane antigen.

This invention provides a method of detecting a nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen and a method of detecting a prostate tumor cell in a subject.

Lastly, this invention provides a pharmaceutical composition comprising a compound in a therapeutically effective amount and a pharmaceutically acceptable carrier and a method of making prostate cells susceptible to a cytotoxic agent.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides an isolated nucleic acid encoding an alternatively spliced human prostate-specific membrane (PSM') antigen. As defined herein "nucleic acid encoding an alternatively spliced prostate-specific membrane (PSM') antigen" means nucleic acid encoding a prostate-specific membrane antigen which contains a deletion in the DNA sequence encoding prostate specific membrane antigen between nucleotide 115 and 380. In one embodiment the isolated nucleic acid encodes the alternatively spliced human prostate-specific membrane antigen as set forth in Figure 55.

This invention further provides an isolated mammalian genomic DNA molecule which encodes an alternatively spliced prostate-specific membrane antigen. This invention further provides an isolated mammalian DNA molecule of an isolated mammalian nucleic acid molecule encoding an alternatively spliced prostate-specific membrane antigen. This invention also provides an isolated mammalian cDNA molecule encoding a mammalian alternatively spliced prostate-specific membrane antigen. This invention provides an isolated mammalian RNA molecule encoding a mammalian alternatively spliced prostate-specific membrane antigen.

This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of PSM' antigen, but which should not produce phenotypic changes. Alternatively, this invention also encompasses DNAs and cDNAs which hybridize to the DNA and cDNA of the subject invention. Hybridization methods are well known to those of skill in the art.

This invention also provides a nucleic acid molecule of

at least 15 nucleotides capable of specifically hybridizing with a sequence of a nucleic acid molecule encoding the prostate-specific membrane antigen. This molecule may either be a DNA or RNA molecule.

This invention provides a nucleic acid sequence of at least 15 nucleotides capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located between nucleotide 115 and nucleotide 380.

The nucleic acid molecule capable of specifically hybridizing with a sequence of a nucleic acid molecule encoding the prostate-specific membrane antigen can be used as a probe. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes PSM antigen into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the PSM antigen molecule downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized PSM antigen fragment where it contains an upstream promoter in the presence of the appropriate

RNA polymerase.

For example, high stringent hybridization conditions are selected at about 5° C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents, ie. salt or formamide concentration, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one. For Example high stringency may be attained for example by overnight hybridization at about 68°C in a 6x SSC solution, washing at room temperature with 6x SSC solution, followed by washing at about 68°C in a 6x SSC in a 0.6x SSX solution.

Hybridization with moderate stringency may be attained for example by: 1) filter pre-hybridizing and hybridizing with a solution of 3x sodium chloride, sodium citrate (SSC), 50% formamide, 0.1M Tris buffer at Ph 7.5, 5x Denhardt's solution; 2.) pre-hybridization at 37°C for 4 hours; 3) hybridization at 37°C with amount of labelled probe equal to 3,000,000 cpm total for 16 hours; 4) wash in 2x SSC and 0.1% SDS solution; 5) wash 4x for 1 minute each at room temperature at 4x at 60°C for 30 minutes each; and 6) dry and expose to film.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

Moreover, the isolated mammalian nucleic acid molecules encoding a mammalian prostate-specific membrane antigen and the alternatively spliced PSM' are useful for the development of probes to study the tumorigenesis of prostate cancer.

The nucleic acid molecules synthesized above may be used to detect expression of a PSM' antigen by detecting the presence of mRNA coding for the PSM antigen. Total mRNA from the cell may be isolated by many procedures well known to a person of ordinary skill in the art. The hybridizing conditions of the labelled nucleic acid molecules may be determined by routine experimentation well known in the art. The presence of mRNA hybridized to the probe may be determined by gel electrophoresis or other methods known in the art. By measuring the amount of the hybrid made, the expression of the PSM and PSM' antigen by the cell can be determined. The labeling may be radioactive. For an example, one or more radioactive nucleotides can be incorporated in the nucleic acid when it is made.

In one embodiment of this invention, nucleic acids are extracted by precipitation from lysed cells and the

mRNA is isolated from the extract using an oligo-dT column which binds the poly-A tails of the mRNA molecules. The mRNA is then exposed to radioactively labelled probe on a nitrocellulose membrane, and the probe hybridizes to and thereby labels complementary mRNA sequences. Binding may be detected by luminescence autoradiography or scintillation counting. However, other methods for performing these steps are well known to those skilled in the art, and the discussion above is merely an example.

The probes are also useful for in-situ hybridization or in order to locate tissues which express this gene, or for other hybridization assays for the presence of this gene or its mRNA in various biological tissues. The in-situ hybridization using a labelled nucleic acid molecule is well known in the art. Essentially, tissue sections are incubated with the labelled nucleic acid molecule to allow the hybridization to occur. The molecule will carry a marker for the detection because it is "labelled", the amount of the hybrid will be determined based on the detection of the amount of the marker and so will the expression of PSM antigen.

This invention further provides isolated PSM' antigen nucleic acid molecule operatively linked to a promoter of RNA transcription. The isolated PSM' antigen sequence can be linked to vector systems. Various vectors including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses are well known to ordinary skilled practitioners. This invention further provides a vector which comprises the isolated nucleic acid molecule encoding for the PSM' antigen.

As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme

to create complementary ends on both molecules which base pair with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other means are also available and known to an ordinary skilled practitioner.

Plasmid, p55A-PSM, was deposited on August 14, 1992 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, p55A-PSM, was accorded ATCC Accession Number 75294.

This invention further provides a host vector system for the production of a polypeptide having the biological activity of the alternatively spliced prostate-specific membrane antigen. These vectors may be transformed into a suitable host cell to form a host cell vector system for the production of a polypeptide having the biological activity of PSM' antigen.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such

vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the PSM antigen.

This invention further provides an isolated DNA or cDNA molecule described hereinabove wherein the host cell is selected from the group consisting of bacterial cells (such as E.coli), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

This invention provides an isolated polypeptide having the biological activity of an alternatively spliced prostate-specific membrane antigen.

This invention further provides a method of producing a polypeptide having the biological activity of the prostate-specific membrane antigen which comprising growing host cells of a vector system containing the PSM' antigen sequence under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

This invention provides a mammalian cell comprising a DNA molecule encoding a mammalian PSM' antigen, such as a mammalian cell comprising a plasmid adapted for expression in a mammalian cell, which comprises a DNA molecule encoding a mammalian PSM' antigen and the regulatory elements necessary for expression of the DNA in the mammalian cell so located relative to the DNA encoding the mammalian PSM' antigen as to permit expression thereof.

Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH3T3, CHO cells, HeLa cells, Ltk$^-$ cells, Cos cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, electroporation or DNA encoding the mammalian PSM antigen may be otherwise introduced into mammalian cells, e.g., by microinjection, to obtain mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding a mammalian PSM antigen.

This invention further provides ligands bound to the mammalian PSM' antigen.

This invention also provides a therapeutic agent comprising a ligand identified by the above-described method and a cytotoxic agent conjugated thereto. The cytotoxic agent may either be a radioisotope or a toxin. Examples of radioisotopes or toxins are well known to one of ordinary skill in the art.

This invention also provides a method of imaging prostate cancer in human patients which comprises administering to the patients at least one ligand identified by the above-described method, capable of binding to the cell surface of the prostate cancer cell and labelled with an imaging agent under conditions permitting formation of a complex between the ligand and the cell surface PSM' antigen. This invention further provides a composition comprising an effective imaging agent of the PSM' antigen ligand and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to one of ordinary skill in the art. For an example, such a pharmaceutically acceptable carrier can be physiological saline.

Also provided by this invention is a purified mammalian PSM' antigen. As used herein, the term "purified alternatively spliced prostate-specific membrane antigen" shall mean isolated naturally-occurring prostate-specific membrane antigen or protein (purified from nature or manufactured such that the primary, secondary and tertiary conformation, and posttranslational modifications are identical to naturally-occurring material) as well as non-naturally occurring polypeptides having a primary structural conformation (i.e. continuous sequence of amino acid residues). Such polypeptides include derivatives and analogs.

This invention provides an isolated nucleic acid comprising a promoter sequence normally associated with the transcription of a gene encoding a human prostate-specific membrane antigen. In one embodiment regulatory elements are set forth in Figure 15. In another embodiment the promoter is between nucleotide -1 to -641 of Figure 15A.

This invention provides a method to identify such natural ligand or other ligand which can bind to the PSM' antigen. A method to identify the ligand comprises a) coupling the purified mammalian PSM' antigen to a solid matrix, b) incubating the coupled purified mammalian PSM' protein with the potential ligands under the conditions permitting binding of ligands and the purified PSM' antigen; c) washing the ligand and coupled purified mammalian PSM' antigen complex formed in b) to eliminate the nonspecific binding and impurities and finally d) eluting the ligand from the bound purified mammalian PSM' antigen. The techniques of coupling proteins to a solid matrix are well known in the art. Potential ligands may

either be deduced from the structure of mammalian PSM' by other empirical experiments known by ordinary skilled practitioners. The conditions for binding may also easily be determined and protocols for carrying such experimentation are known to those skilled in the art. The ligand-PSM' antigen complex will be washed. Finally, the bound ligand is eluted and characterized. Standard ligands characterization techniques are well known in the art.

The above method may also be used to purify ligands from any biological source. For purification of natural ligands in the cell, cell lysates, serum or other biological samples will be used to incubate with the mammalian PSM' antigen bound on a matrix. Specific natural ligand will then be identified and purified as above described.

With the protein sequence information, antigenic areas may be identified and antibodies directed against these areas may be generated and targeted to the prostate cancer for imaging the cancer or therapies.

This invention provides an antibody directed against the amino acid sequence of a mammalian PSM' antigen.

This invention provides a method to select specific regions on the PSM' antigen to generate antibodies. The protein sequence may be determined from the PSM' DNA sequence. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer of the cell membrane, while hydrophilic regions are located on the

cell surface, in an aqueous environment. Usually, the hydrophilic regions will be more immunogenic than the hydrophobic regions. Therefore the hydrophilic amino acid sequences may be selected and used to generate antibodies specific to mammalian PSM antigen. For an example, hydrophilic sequences of the human PSM antigen shown in hydrophilicity plot may be easily selected. The selected peptides may be prepared using commercially available machines. As an alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen.

Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be produced by <u>in vitro</u> techniques known to a person of ordinary skill in the art. These antibodies are useful to detect the expression of mammalian PSM antigen in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

In one embodiment, peptides Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID No.), Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No.) and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.) of human PSM antigen are selected.

This invention further provides polyclonal and monoclonal antibody(ies) against peptides Asp-Glu-Leu-Lys-Ala-Glu (SEQ ID No.), Asn-Glu-Asp-Gly-Asn-Glu (SEQ ID No.) and Lys-Ser-Pro-Asp-Glu-Gly (SEQ ID No.).

This invention provides a method of imaging prostate cancer in human patients which comprises administering to the patient the monoclonal antibody directed against the peptide of the mammalian PSM' antigen capable of binding to the cell surface of the prostate cancer cell and labeled with an imaging agent under conditions permitting formation of a complex between the monoclonal antibody and the cell surface prostate-specific membrane antigen. The imaging agent is a radioisotope such as $Indium^{111}$.

This invention further provides a prostate cancer specific imaging agent comprising the antibody directed against PSM' antigen and a radioisotope conjugated thereto.

This invention also provides a composition comprising an effective imaging amount of the antibody directed against the PSM' antigen and a pharmaceutically acceptable carrier. The methods to determine effective imaging amounts are well known to a skilled practitioner. One method is by titration using different amounts of the antibody.

In addition to the standard pharmacophores that can be added to know structures, with the PSM transfectants one can identify potential ligands from combinatorial libraries that might not have been otherwise predicted such combinatorial libraries can be synthetic, peptide, or RNA based.

This invention further provides an immunoassay for measuring the amount of the prostate-specific membrane antigen in a biological sample comprising steps of a) contacting the biological sample with at least one antibody directed against the PSM' antigen to form a complex with said antibody and the prostate-specific

membrane antigen, and b) measuring the amount of the prostate-specific membrane antigen in said biological sample by measuring the amount of said complex. One example of the biological sample is a serum sample.

This invention provides a method to purify mammalian prostate-specific membrane antigen comprising steps of a) coupling the antibody directed against the PSM' antigen to a solid matrix; b) incubating the coupled antibody of a) with lysate containing prostate-specific membrane antigen under the condition which the antibody and prostate membrane specific can bind; c) washing the solid matrix to eliminate impurities and d) eluting the prostate-specific membrane antigen from the coupled antibody.

This invention also provides a transgenic nonhuman mammal which comprises the isolated nucleic acid molecule encoding a mammalian PSM' antigen. This invention further provides a transgenic nonhuman mammal whose genome comprises antisense DNA complementary to DNA encoding a mammalian prostate-specific membrane antigen so placed as to be transcribed into antisense mRNA complementary to mRNA encoding the prostate-specific membrane antigen and which hybridizes to mRNA encoding the prostate specific antigen thereby reducing its translation.

Animal model systems which elucidate the physiological and behavioral roles of mammalian PSM' antigen are produced by creating transgenic animals in which the expression of the PSM' antigen is either increased or decreased, or the amino acid sequence of the expressed PSM antigen is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding a mammalian PSM' antigen, by

is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Another use of the PSM antigen sequence is to isolate homologous gene or genes in different mammals. The gene or genes can be isolated by low stringency screening of either cDNA or genomic libraries of different mammals using probes from PSM sequence. The positive clones identified will be further analyzed by DNA sequencing techniques which are well known to an ordinary person skilled in the art. For example, the detection of members of the protein serine kinase family by homology probing.

This invention provides a method of suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells comprising introducing a DNA molecule encoding an alternatively spliced prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell of a subject, in a way that expression of the alternatively spliced prostate specific membrane antigen is under the control of the regulatory element, thereby suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells. The subject may be a mammal or more specifically a human.

In one embodiment, the DNA molecule is operatively linked to a 5' regulatory element forms part of a transfer vector which is inserted into a cell or organism. In addition the vector is capable or replication and expression of the alternatively spliced prostate specific membrane antigen. The DNA molecule can be integrated into a genome of a eukaryotic or prokaryotic cell or in a host cell containing and/or expressing an alternatively spliced prostate specific membrane antigen.

Further, the DNA molecule encoding alternatively spliced prostate specific membrane antigen may be introduced by a bacterial, viral, fungal, animal, or liposomal delivery vehicle. Other means are also available and known to an ordinary skilled practitioner.

Further, the DNA molecule encoding an alternatively spliced prostate specific membrane antigen operatively linked to a promoter or enhancer. A number of viral vectors have been described including those made from various promoters and other regulatory elements derived from virus sources. Promoters consist of short arrays of nucleic acid sequences that interact specifically with cellular proteins involved in transcription. The combination of different recognition sequences and the cellular concentration of the cognate transcription factors determines the efficiency with which a gene is transcribed in a particular cell type.

Examples of suitable promoters include a viral promoter. Viral promoters include: adenovirus promoter, an simian virus 40 (SV40) promoter, a cytomegalovirus (CMV) promoter, a mouse mammary tumor virus (MMTV) promoter, a Malony murine leukemia virus promoter, a murine sarcoma virus promoter, and a Rous

microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these PSM' antigen sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native PSM antigen but does express, for example, an inserted mutant PSM antigen, which has replaced the native PSM antigen in the animal's genome by recombination, resulting in under expression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added PSM antigens, resulting in over expression of the PSM antigens.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as Me medium (16). DNA or cDNA encoding a mammalian PSM antigen is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected

sarcoma virus promoter.

Further, another suitable promoter is a heat shock promoter. Additionally, a suitable promoter is a bacteriophage promoter. Examples of suitable bacteriophage promoters include but not limited to, a T7 promoter, a T3 promoter, an SP6 promoter, a lambda promoter, a baculovirus promoter.

Also suitable as a promoter is an animal cell promoter such as an interferon promoter, a metallothionein promoter, an immunoglobulin promoter. A fungal promoter is also a suitable promoter. Examples of fungal promoters include but are not limited to, an ADC1 promoter, an ARG promoter, an ADH promoter, a CYC1 promoter, a CUP promoter, an ENO1 promoter, a GAL promoter, a PHO promoter, a PGK promoter, a GAPDH promoter, a mating type factor promoter. Further, plant cell promoters and insect cell promoters are also suitable for the methods described herein.

This invention provides a method of suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells, comprising introducing a DNA molecule encoding an alternatively spliced prostate specific membrane antigen operatively linked to a 5' regulatory element coupled with a therapeutic DNA into a tumor cell of a subject, thereby suppressing or modulating metastatic ability of prostate tumor cells, prostate tumor growth or elimination of prostate tumor cells. The subject may be a mammal or more specifically a human.

Further, the therapeutic DNA which is coupled to the DNA molecule encoding a prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell may code for a cytokine, viral

antigen, or a pro-drug activating enzyme. Other means are also available and known to an ordinary skilled practitioner.

In addition, this invention provides a prostate tumor cell, comprising a DNA molecule isolated from mammalian nucleic acid encoding an alternatively spliced mammalian prostate-specific membrane antigen under the control a 5' regulatory element.

As used herein, DNA molecules include complementary DNA (cDNA), synthetic DNA, and genomic DNA.

This invention provides a therapeutic vaccine for preventing human prostate tumor growth or stimulation of prostate tumor cells in a subject, comprising administering an effective amount to the prostate cell, and a pharmaceutical acceptable carrier, thereby preventing the tumor growth or stimulation of tumor cells in the subject. Other means are also available and known to an ordinary skilled practitioner.

This invention provides a method of detecting hematogenous micrometastic tumor cells of a subject, comprising (A) performing nested polymerase chain reaction (PCR) on blood, bone marrow or lymph node samples of the subject using the prostate specific membrane antigen primers or alternatively spliced prostate specific antigen primers, and (B) verifying micrometastases by DNA sequencing and Southern analysis, thereby detecting hematogenous micrometastic tumor cells of the subject. The subject may be a mammal or more specifically a human.

The micrometastatic tumor cell may be a prostatic cancer and the DNA primers may be derived from prostate specific antigen. Further, the subject may be

administered with simultaneously an effective amount of hormones, so as to increase expression of prostate specific membrane antigen. Further, growth factors or cytokine may be administered in separately or in conjunction with hormones. Cytokines include, but are not limited to: transforming growth factor beta, epidermal growth factor (EGF) family, fibroblast growth factors, hepatocyte growth factor, insulin-like growth factors, B-nerve growth factor, platelet-derived growth factor, vascular endothelial growth factor, interleukin 1, IL-1 receptor antagonist, interleukin 2, interleukin 3, interleukin 4, interleukin 5, interleukin 6, IL-6 soluble receptor, interleukin 7, interleukin 8, interleukin 9, interleukin 10, interleukin 11, interleukin 12, interleukin 13, angiogenin, chemokines, colony stimulating factors, granulocyte-macrophage colony stimulating factors, erythropoietin, interferon, interferon gamma, leukemia inhibitory factor, oncostatin M, pleiotrophin, secretory leukocyte protease inhibitor, stem cell factor, tumor necrosis factors, adhesion molecule, and soluble tumor necrosis factor (TNF) receptors.

This invention provides a method of abrogating the mitogenic response due to transferrin, comprising introducing a DNA molecule encoding prostate specific membrane antigen operatively linked to a 5' regulatory element into a tumor cell, the expression of which gene is directly associated with a defined pathological effect within a multicellular organism, thereby abrogating mitogen response due to transferrin. The tumor cell may be a prostate cell.

This invention provides a method of determining prostate cancer progression in a subject which comprises: a) obtaining a suitable prostate tissue sample; b) extracting RNA from the prostate tissue

sample; c) performing a RNAse protection assay on the RNA thereby forming a duplex RNA-RNA hybrid; d) detecting PSM and PSM' amounts in the tissue sample; e) calculating a PSM/PSM' tumor index, thereby determining prostate cancer progression in the subject. In-situ hyribridization may be performed in conjunction with the above detection method.

This invention provides a method of detecting prostate cancer in a subject which comprises: (a) obtaining from a subject a prostate tissue sample; (b) treating the tissue sample so as to separately recover nucleic acid molecules present in the prostate tissue sample; (c) contacting the resulting nucleic acid molecules with multiple pairs of single-stranded labeled oligonucleotide primers, each such pair being capable of specifically hybridizing to the tissue sample, under hybridizing conditions; (d) amplifying any nucleic acid molecules to which a pair of primers hybridizes so as to obtain a double-stranded amplification product; (e) treating any such double-stranded amplification product so as to obtain single-stranded nucleic acid molecules therefrom; (f) contacting any resulting single-stranded nucleic acid molecules with multiple single-stranded labeled oligonucleotide probes, each such probe containing the same label and being capable of specifically hybridizing with such tissue sample, under hybridizing conditions; (g) contacting any resulting hybrids with an antibody to which a marker is attached and which is capable of specifically forming a complex with the labeled-probe, when the probe is present in such a complex, under complexing conditions; and (h) detecting the presence of any resulting complexes, the presence thereof being indicative of prostate cancer in a subject.

This invention provides a method of enhancing antibody

based targeting of PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient b-FGF in sufficient amount to cause upregulation of PSM' expression.

This invention provides a method of enhancing antibody based targeting of PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient TGF in sufficient amount to cause upregulation of PSM expression or PSM'.

This invention provides a method of enhancing antibody based targeting of PSM' in prostate tissue for diagnosis or therapy of prostate cancer comprising administering to a patient EGF in sufficient amount to cause upregulation of PSM' expression.

This invention provides a method of detecting in a sample the presence of a nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen which comprises: a) obtaining a suitable sample; b) extracting RNA from the sample; c) contacting the RNA with reverse transcriptase under suitable conditions to obtain a cDNA; d) contacting the cDNA under hybridizing conditions with two oligonucleotide primers, i) the first primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 3' of nucleotide 114 of such DNA sequence, with the proviso that the 3' end of the primer does not hybridize to any sequence located 5' of nucleotide 114, and ii) the second primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 5' of nucleotide 381 of such DNA sequence, with the proviso that the 5' end of the primer does not hybridize to any

sequence located 3' of nucleotide 381; d) amplifying any cDNA to which the primers hybridize to so as to obtain amplification product; e) determining the size of the amplification product; f) comparing the size of the amplification product to the size of the amplification product known to be obtained using the same primers with a non alternatively spliced human prostate specific membrane antigen, wherein a smaller amplification product is indicative of the presence of the alternatively spliced prostate specific membrane antigen, so as to thereby detect the presence of the alternatively spliced human prostate-specific membrane antigen in the sample.

In one embodiment the suitable sample may be any bodily tissue of fluid which includes but is not limited to: blood, bone marrow, and lymph nodes.

In one embodiment the primers are at least 14-25 nucleotides in length. In another embodiment the primers are at least 15 nucleotide in length. In another embodiment the primers are 15 nucleotides in length. In another embodiment multiple primers are used. Construction of primers which hybridize and hybridizing conditions are known to those skilled in the art. For example, based on Figure 18 one skilled in the art may construct primers which hybridize to the prostate specific membrane antigen before nucleotide 114 and after nucleotide 381.

Further, a method of determining the amount of the amplification product or products (i.e. 2 or more bands) as well as the ratio of each product is known to those skilled in the art. For example, the amount of prostate specific membrane antigen or alternatively spliced prostate specific membrane antigen may be determined by density, binding radiolabled probes,

autoradiography, UV spectrography, spectrophotometer, optical scan , and phospho-imaging.

This invention provides a method of detecting a prostate tumor cell in a subject which comprises: which comprises: a) obtaining a suitable sample; b) extracting RNA from the sample; c) contacting the RNA with reverse transcriptase under suitable conditions to obtain a cDNA; d) contacting the cDNA under hybridizing conditions with two oligonucleotide primers, i) the first primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 3' of nucleotide 114 of such DNA sequence, with the proviso that the 3' end of the primer does not hybridize to any sequence located 5' of nucleotide 114, and ii) the second primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 5' of nucleotide 381 of such DNA sequence, with the proviso that the 5' end of the primer does not hybridize to any sequence located 3' of nucleotide 381; d) amplifying any cDNA to which the primers hybridize to so as to obtain amplification product; e) determining the amount of the amplification product; f) comparing the amount of the amplification product to the amount of the amplification product known to be obtained using the same primers with a non alternatively spliced human prostate specific membrane antigen, wherein a greater amount of the prostate specific membrane antigen is indicative of a prostate tumor cell in the subject, so as to thereby detect prostate tumor cell in the subject.

In PCR techniques, oligonucleotide primers complementary to the two 3' borders of the DNA of the prostate specific membrane (PSM) antigen to be

amplified are synthesized. The polymerase chain reaction is then carried out using the two primers. See *PCR Protocols: A Guide to Methods and Applications*. Hybridization of PSM antigen DNA to the above nucleic acid probes can be performed by a Southern blot under stringent hybridization conditions as described herein.

Oligonucleotides for use as probes or PCR primers are chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage and Carruthers using an automated synthesizer, as described in Needham-VanDevanter. Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson, J.D. and Regnier, F.E. The sequence of the synthetic oligonucleotide can be verified using the chemical degradation method of Maxam, A.M. and Gilbert, W.

Accepted means for conducting hybridization assays are known and general overviews of the technology can be had from a review of: *Nucleic Acid Hybridization: A Practical Approach; Hybridization of Nucleic Acids Immobilized on Solid Supports; Analytical Biochemistry* and Innis *et al., PCR Protocols*.

If PCR is used in conjunction with nucleic acid hybridization, primers are designed to target a specific portion of the nucleic acid of DNA of the PSM antigen. From the information provided herein, those of skill in the art will be able to select appropriate specific primers.

It will be apparent to those of ordinary skill in the art that a convenient method for determining whether a probe is specific for PSM antigen or PSM' antigen utilizes a Southern blot (or Dot blot). Briefly, to

identify a target specific probe DNA is isolated from the PSM or PSM' antigen. Test DNA is transferred to a solid (e.g., charged nylon) matrix. The probes are labelled following conventional methods. Following denaturation and/or prehybridization steps known in the art, the probe is hybridized to the immobilized DNAs under stringent conditions. Stringent hybridization conditions will depend on the probe used and can be estimated from the calculated T_m (melting temperature) of the hybridized probe (see, *e.g.*, Sambrook for a description of calculation of the T_m). For radioactively-labeled DNA or RNA probes an example of stringent hybridization conditions is hybridization in a solution containing denatured probe and 5x SSC at 65°C for 8-24 hours followed by washes in 0.1x SSC, 0.1% SDS (sodium dodecyl sulfate) at 50-65°C. In general, the temperature and salt concentration are chosen so that the post hybridization wash occurs at a temperature that is about 5°C below the T_M of the hybrid. Thus for a particular salt concentration the temperature may be selected that is 5°C below the T_M or conversely, for a particular temperature, the salt concentration is chosen to provide a T_M for the hybrid that is 5°C warmer than the wash temperature. Following stringent hybridization and washing, a probe that hybridizes to the PSM antigen or PSM' antigen as evidenced by the presence of a signal associated with the appropriate target and the absence of a signal from the non-target nucleic acids, is identified as specific. It is further appreciated that in determining probe specificity and in utilizing the method of this invention a certain amount of background signal is typical and can easily be distinguished by one of skill from a specific signal. Two fold signal over background is acceptable.

This invention provides a therapeutic agent comprising

antibodies or ligand(s) directed against PSM' antigen and a cytotoxic agent conjugated thereto or antibodies linked enzymes which activate prodrug to kill the tumor. The cytotoxic agent may either be a radioisotope or toxin.

This invention provides a compound comprising a conjugate of a cytotoxic agent and one or more amino acid residues, wherein each amino acid residue is glutamate or aspartate. In one embodiment the amino acid residues alternate.

Examples of cytotoxic chemotherapeutic agents or antineolastic agents) include, but are not limited to the following: Antimetaboloites: Denopterin, Edatrexate, Piritrexim, Pteropterin, Tomudex, Tremetrexate, Cladribine, Fludarabine, 6-Mercaptopurine, Thiamiprine, Thioguanine, Ancitabine, Azacitidine, 6-Azauridine, Carmofur, Cytarabine, Doxifluride, Emitefur, Enocitabine, Floxuridine, Fluoroucit, Gemcitabine, and Tegafur.

Alkaloids: Docetaxel, Etoposide, Irinotecan, Paclitaxel, Teniposide, Topotecan, VinblastinE, Vincristine,and Vindesine.

Alkylating agents: Alkyl Sulfonates: Busulfan, Improsulfan, Piposulfan, Aziridines, Benzodepa, Carboquone, Meuredepa, Uredepa, Ethylenimines and Methylmelamines, Altretamine, Triethylenemelamine, Triethylenophosphoramide, Triethylenethiophosphoramide, Chlorambucil, Chlornaphazine, Cyclophosphamide, Estramustine, Ifosfamide, Mechlorethamine, Mechlorethamine Oxide Hydrochloride, Melphalan, Novembiechin, Perfosfamide, Phenesterine, Prednimustine, Trofosfamide, Uracil Mustard, Carmustine, Chlorozotocin, Fotemustine, Lomustine,

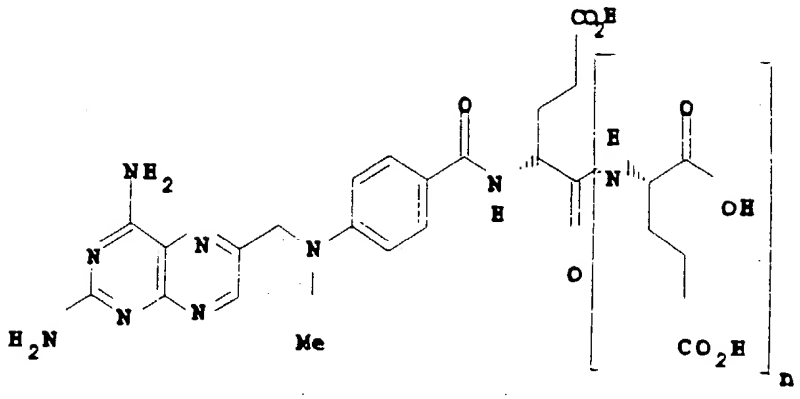
Nimustine, Ranimustine, Dacarbazine, Mannomustine, Mitbronitol, Mitolactol, Pipobroman, Temozolomide, Antibiotics and Analogs: Aclacinomycins, Actinomycin, Anthramycin, Azaserine, Bleomycins, Cactinomycin, Carubicin, Carzinophilin, Chromomycins, Dactinomycin, Caunorubicin, 6-Diazo-5-oxo-L-norleucine, Doxorubicin, Epirubicin, Idarubicin, Menogaril, Mitomycins, Mycophenolic Acid, Nogalamycin, Olivomycins, Peplomycin, Pirarubicin, Plicamycin, Porfiromycin, Puromycin, Streptonigrin, Streptozocin, Tubercidin, Zinostatin, Zorubicin, and L-Asparaginase.

Immunodulators: Interferon, Interferon-B, Interferon-Y, Interleukin-2, Lentinan, Propagermanium, PSK, Roquinimex, Sizofran, and Ubenimex. Platinum complexes: Carboplatin, Cisplatin, Miboplatin, and Oxaliplatin.

Others: Aceglatone, Amsacrine, Bisantrene, Defoosfamide, Demecolcine, Diaziqone, Eflornithine, Eliptinium Acetate, Etoglucid, Fenertinide, Gallium Nitrate, Hydroxyurea, Lonidamine, Miltefosine, Mitoguazone, Mitoxantrone, Mopidamol, Nitracirine, Pentostatin, Phenamet, Podophyllinic Acid 2-Ethyl-hydrazide, Procarbazine, Razoxane, Sobuzoxane, Spirogermanium, Tenuazonic Acid, Triaziquone, Urethan, Calusterone, Dromostanolone, Epitiostanol, Mepitiostane, Testolactone, Amiglutehimide, Mitotane, Trilostane, Droloxifene, Tamoxifen, Toremifene, Aminoglutethimide, Anastrozole, Fadrozole, Formestane, Letrozole, Fosfestrol, Hexestrol, Polyestradiol Phosphate, Buserlin, Goserlin, Leuprolide, Triptorelin, Chlormadinone Acetate, Medroxyprogesterone, Megerstrol Acetate, Melengestrol, Porfimer Sodium, Americium, Chromic Phosphate, Radioactive Cobalt, I-Ehtiodized Oil, Gold, Radioactive, Colloidal, Iobenguane, Radium, Radon, Sodium Iodide, Sodium Phosphate, Radioactive, Batimastat, Folinic Acid, Amifostine, Etanidazole,

Etamidozole, and Mesna.

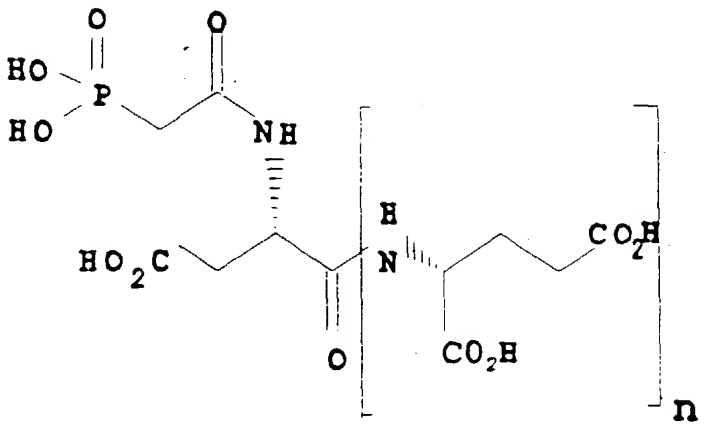
This invention provides a compound, wherein the compound has the structure:



wherein n is an integer from 1-10 inclusive.

In one embodiment glutamate may be in L or D to form either 4-amino-N^{10}-methyl pteroyl-L-glutamate or 4-amino-N^{10}-methyl pteroyl-D-glutamate. In another embodiment aspartate may substitute the glutamate to form 4-amino-N^{10}-methyl pteroyl-L-aspartate. In another embodiment aspartate may substitute the glutamate to form 4-amino-N^{10}-methyl pteroyl-D-aspartate. In another embodiment the 4-amino-N^{10}-methyl pteroyl may have alternating glutamate or aspartat moieties. The glutamate or aspartate are bound to the methotrexate at the alpha carbon position of methotrexate.

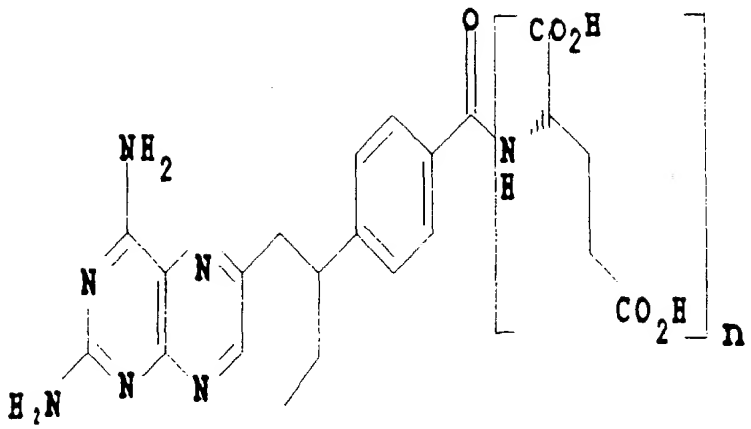
This invention provides a compound, wherein the compound has the structure:



wherein n is an integer from 1-10 inclusive.

In one embodiment glutamate may be in the L or D to form either N-phosphonoacetyl-L-aspartyl (PALA)-glutamate or N-phsophonoacetyl-D-aspartyl-glutamate. In another embodiment aspartate may substitute the glutamate to form N-phsophonoacetyl-L-aspartyl-aspartate. In another embodiment the 4-amino-N^{10}-methyl pteroyl may have alternating glutamate or aspartate moieties.

This invention provides a compound, wherein the compound has the structure:



wherein n is an integer from 1-10 inclusive.

In one embodiment glutamate may be in the L or D to form either 4-amino-10-ethyl-10-deazapteroyl (EDAM) - L-glutamate or 4-amino-10-ethyl-10-deazapteroyl-D-glutamate. In another embodiment aspartate may substitute the glutamate to form 4-amino-10-ethyl-10-deazapteroyl-L-aspartate. In another embodiment the 4-amino-10-ethyl-10-deazapteroyl may have alternating glutamate or aspartat moieties.

This invention provides a pharmaceutical composition comprising any of the above compounds in a therapeutically effective amount and a pharmaceutically acceptable carrier.

This invention provides a method of making prostate cells susceptible to a cytotoxic agent, which comprises contacting the prostate cells with any of the above compounds in an amount effective to render the prostate cells susceptible to the cytotoxic chemotherapeutic

agent.

This invention provides a pharmaceutical composition comprising an effective amount the alternatively spliced PSM' and a carrier or diluent. Further, this invention provides a method for administering to a subject, preferably a human, the pharmaceutical composition. Further, this invention provides a composition comprising an amount of the alternatively spliced PSM' and a carrier or diluent. Specifically, this invention may be used as a food additive.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each subject.

In one embodiment the therapeutic effective amount is 100-10,000 mg/m^2 IV with rescue. In another embodiment the therapeutic effective amount is 300-1000 mg/m^2 IV or continuous infusion. In another embodiment the therapeutic effective amount is 100 mg/m^2 IV continuous infusion. In another embodiment the therapeutic effective amount is 40-75 mg/m^2 rapidly. In another embodiment the therapeutic effective amount is 30 mg/m^2 for 3 days by continuous IV.

Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration.

As used herein administration means a method of administering to a subject. Such methods are well

known to those skilled in the art and include, but are not limited to, administration topically, parenterally, orally, intravenously, intramuscularly, subcutaneously or by aerosol. Administration of PSM may be effected continuously or intermittently.

The pharmaceutical formulations or compositions of this invention may be in the dosage form of solid, semi-solid, or liquid such as, *e.g.*, suspensions, aerosols or the like. Preferably the compositions are administered in unit dosage forms suitable for single administration of precise dosage amounts. The compositions may also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological saline, Ringer's solution, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants; or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like. Effective amounts of such diluent or carrier are those amounts which are effective to obtain a pharmaceutically acceptable formulation in terms of solubility of components, or biological activity, etc

This invention also provides a method of detecting a subject with cancer comprising a) contacting a cell of the neo-vasculature of a subject with a ligand which binds to the extraccelular domain of the PSM antigen under conditions permitting formation of a complex; and b) detecting the complex with a labelled imaging

agent, thereby detecting a subject with cancer.

In one embodiment the cancer is, but is not limited to: kidney, colon, or bladder. In one embodiment the ligand is CYT-356. In another embodiment the ligand is any antibody, monoclonal or polyclonal which binds to the extracellular domain of PSM antigen. In one embodiment the cells of endothelial cells of the neo-vasculature of a subject with cancer.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

EXAMPLE 1:

EXPRESSION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN

A 2.65 kb complementary DNA encoding PSM was cloned. Immunohistochemical analysis of the LNCaP, DU-145, and PC-3 prostate cancer cell lines for PSM expression using the 7E11-C5.3 antibody reveals intense staining in the LNCaP cells, with no detectable expression in both the DU-145 and PC-3 cells. Coupled *in-vitro* transcription/ translation of the 2.65 kb full-length PSM cDNA yields an 84 kDa protein corresponding to the predicted polypeptide molecular weight of PSM. Post-translational modification of this protein with pancreatic canine microsomes yields the expected 100 kDa PSM antigen. Following transfection of PC-3 cells with the full-length PSM cDNA in a eukaryotic expression vector applicant's detect expression of the PSM glycoprotein by Western analysis using the 7E11-C5.3 monoclonal antibody. Ribonuclease protection analysis demonstrates that the expression of PSM mRNA is almost entirely prostate-specific in human tissues. PSM expression appears to be highest in hormone-deprived states and is hormonally modulated by steroids, with DHT down regulating PSM expression in the human prostate cancer cell line LNCaP by 8-10 fold, testosterone down regulating PSM by 3-4 fold, and corticosteroids showing no significant effect. Normal and malignant prostatic tissues consistently show high PSM expression, whereas heterogeneous, and at times absent, from expression of PSM in benign prostatic hyperplasia. LNCaP tumors implanted and grown both orthotopically and subcutaneously in nude mice, abundantly express PSM providing an excellent *in-vivo* model system to study the regulation and modulation of

PSM expression.

Materials and Methods:

Cells and Reagents: The LNCaP, DU-145, and PC-3 cell lines were obtained from the American Type Culture Collection. Details regarding the establishment and characteristics of these cell lines have been previously published. Unless specified otherwise, LNCaP cells were grown in RPMI 1640 media supplemented with L-glutamine, nonessential amino acids, and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD.) in a CO_2 incubator at 37C. DU-145 and PC-3 cells were grown in minimal essential medium supplemented with 10% fetal calf serum. All cell media were obtained from the MSKCC Media Preparation Facility. Restriction and modifying enzymes were purchased from Gibco-BRL unless otherwise specified.

Immunohistochemical Detection of PSM: Avidin-biotin method of detection was employed to analyze prostate cancer cell lines for PSM antigen expression. Cell cytospins were made on glass slides using $5x10^4$ cells/100ul per slide. Slides were washed twice with PBS and then incubated with the appropriate suppressor serum for 20 minutes. The suppressor serum was drained off and the cells were incubated with diluted 7E11-C5.3 (5g/ml) monoclonal antibody for 1 hour. Samples were then washed with PBS and sequentially incubated with secondary antibodies for 30 minutes and with avidin-biotin complexes for 30 minutes. Diaminobenzidine served as the chromogen and color development followed by hematoxylin counterstaining and mounting. Duplicate cell cytospins were used as controls for each experiment. As a positive control, the anti-cytokeratin monoclonal antibody CAM 5.2 was used following the same procedure described above. Human EJ

bladder carcinoma cells served as a negative control.

In-Vitro Transcription/Translation of PSM Antigen: Plasmid 55A containing the full length 2.65 kb PSM cDNA in the plasmid pSPORT 1 (Gibco-BRL) was transcribed in-vitro using the Promega TNT system (Promega Corp. Madison, WI). T7 RNA polymerase was added to the cDNA in a reaction mixture containing rabbit reticulocyte lysate, an amino acid mixture lacking methionine, buffer, and ^{35}S-Methionine (Amersham) and incubated at 30C for 90 minutes. Post-translational modification of the resulting protein was accomplished by the addition of pancreatic canine microsomes into the reaction mixture (Promega Corp. Madison, WI.). Protein products were analyzed by electrophoresis on 10% SDS-PAGE gels which were subsequently treated with Amplify autoradiography enhancer (Amersham, Arlington Heights, IL.) according to the manufacturers instructions and dried at 80C in a vacuum dryer. Gels were autoradiographed overnight at -70C using Hyperfilm MP (Amersham).

Transfection of PSM into PC-3 Cells: The full length PSM cDNA was subcloned into the pREP7 eukaryotic expression vector (Invitrogen, San Diego, CA.). Plasmid DNA was purified from transformed DH5-alpha bacteria (Gibco-BRL) using Qiagen maxi-prep plasmid isolation columns (Qiagen Inc., Chatsworth, CA.). Purified plasmid DNA (6-10g) was diluted with 900ul of Optimem media (Gibco-BRL) and mixed with 30ul of Lipofectin reagent (Gibco-BRL) which had been previously diluted with 900l of Optimem media. This mixture was added to T-75 flasks of 40-50% confluent PC-3 cells in Optimem media. After 24-36 hours, cells were trypsinized and split into 100mm dishes containing RPMI 1640 media supplemented with 10% fetal calf serum and 1 mg/ml of Hygromycin B (Calbiochem, La

Jolla, CA.). The dose of Hygromycin B used was previously determined by a time course/dose response cytotoxicity assay. Cells were maintained in this media for 2-3 weeks with changes of media and Hygromycin B every 4-5 days until discrete colonies appeared. Colonies were isolated using 6mm cloning cylinders and expanded in the same media. As a control, PC-3 cells were also transfected with the pREP7 plasmid alone. RNA was isolated from the transfected cells and PSM mRNA expression was detected by both RNase Protection analysis (described later) and by Northern analysis.

Western Blot Detection of PSM Expression: Crude protein lysates were isolated from LNCaP, PC-3, and PSM-transfected PC-3 cells as previously described. LNCaP cell membranes were also isolated according to published methods. Protein concentrations were quantitated by the Bradford method using the BioRad protein reagent kit (BioRad, Richmond, CA.). Following denaturation, 20μg of protein was electrophoresed on a 10% SDS-PAGE gel at 25 mA for 4 hours. Gels were electroblotted onto Immobilon P membranes (Millipore, Bedford, MA.) overnight at 4C. Membranes were blocked in 0.15M NaCl/0.01M Tris-HCl (TS) plus 5% BSA followed by a 1 hour incubation with 7E11-C5.3 monoclonal antibody (10μg/ml). Blots were washed 4 times with 0.15M NaCl/0.01M Tris-HCl/0.05% Triton-X 100 (TS-X) and incubated for 1 hour with rabbit anti-mouse IgG (Accurate Scientific, Westbury, N.Y.) at a concentration of 10μg/ml.

Blots were then washed 4 times with TS-X and labeled with ^{125}I-Protein A (Amersham, Arlington Heights, IL.) at a concentration of 1 million cpm/ml. Blots were then washed 4 times with TS-X and dried on Whatman 3MM paper, followed by overnight autoradiography at -70C

using Hyperfilm MP (Amersham).

Orthotopic and Subcutaneous LNCaP Tumor Growth in Nude Mice: LNCaP cells were harvested from sub-confluent cultures by a one minute exposure to a solution of 0.25% trypsin and 0.02% EDTA. Cells were resuspended in RPMI 1640 media with 5% fetal bovine serum, washed and diluted in either Matrigel (Collaborative Biomedical Products, Bedford, MA.) or calcium and magnesium-free Hank's balanced salt solution (HBSS). Only single cell suspensions with greater than 90% viability by trypan blue exclusion were used for in vivo injection. Male athymic Swiss (nu/nu) nude mice 4-6 weeks of age were obtained from the Memorial Sloan-Kettering Cancer Center Animal Facility. For subcutaneous tumor cell injection one million LNCaP cells resuspended in 0.2 mls. of Matrigel were injected into the hindlimb of each mouse using a disposable syringe fitted with a 28 gauge needle. For orthotopic injection, mice were first anesthetized with an intraperitoneal injection of Pentobarbital and placed in the supine position. The abdomen was cleansed with Betadine and the prostate was exposed through a midline incision. 2.5 million LNCaP tumor cells in 0.1 ml. were injected directly into either posterior lobe using a 1 ml disposable syringe and a 28 gauge needle. LNCaP cells with and without Matrigel were injected. Abdominal closure was achieved in one layer using Autoclip wound clips (Clay Adams, Parsippany, N.J.). Tumors were harvested in 6-8 weeks, confirmed histologically by faculty of the Memorial Sloan-Kettering Cancer Center Pathology Department, and frozen in liquid nitrogen for subsequent RNA isolation.

RNA Isolation: Total cellular RNA was isolated from cells and tissues by standard techniques (3 and 17) as well as by using RNAzol B (Cinna/Biotecx, Houston,

TX.). RNA concentrations and quality were assessed by UV spectroscopy on a Beckman DU 640 spectrophotometer and by gel analysis. Human tissue total RNA samples were purchased from Clontech Laboratories, Inc., Palo Alto, CA.

Ribonuclease Protection Assays: A portion of the PSM cDNA was subcloned into the plasmid vector pSPORT 1 (Gibco-BRL) and the orientation of the cDNA insert relative to the flanking T7 and SP6 RNA polymerase promoters was verified by restriction analysis. Linearization of this plasmid upstream of the PSM insert followed by transcription with SP6 RNA polymerase yields a 400 nucleotide antisense RNA probe, of which 350 nucleotides should be protected from RNase digestion by PSM RNA. This probe was used in Figure 20. Plasmid IN-20, containing a 1 kb partial PSM cDNA in the plasmid pCR II (Invitrogen) was also used for riboprobe synthesis. IN-20 linearized with Xmn I (Gibco-BRL) yields a 298 nucleotide anti-sense RNA probe when transcribed using SP6 RNA polymerase, of which 260 nucleotides should be protected from RNase digestion by PSM mRNA. This probe was used in Figures 21 and 22. Probes were synthesized using SP6 RNA polymerase (Gibco-BRL), rNTPs (Gibco-BRL), RNAsin (Promega), and ^{32}P-rCTP (NEN, Wilmington, DE.) according to published protocols (44). Probes were purified over NENSORB 20 purification columns (NEN) and approximately 1 million cpm of purified, radiolabeled PSM probe was mixed with 10μ of each RNA and hybridized overnight at 45C using buffers and reagents from the RPA II kit (Ambion, Austin, TX). Samples were processed as per manufacturer's instructions and analyzed on 5% polyacrilamide/7M urea denaturing gels using Seq ACRYL reagents (ISS, Natick, MA.). Gels were pre-heated to 55C and run for approximately 1-2 hours at 25 watts. Gels were then fixed for 30 minutes in 10% methanol/10%

acetic acid, dried onto Whatman 3MM paper at 80C in a BioRad vacuum dryer and autoradiographed overnight with Hyperfilm MP (Amersham). Quantitation of PSM expression was determined by using a scanning laser densitometer (LKB, Piscataway, NJ.).

Steroid Modulation Experiment: LNCaP cells (2 million) were plated onto T-75 flasks in RPMI 1640 media supplemented with 5% fetal calf serum and grown 24 hours until approximately 30-40% confluent. Flasks were then washed several times with phophate-buffered saline and RPMI medium supplemented with 5% charcoal-extracted serum was added. Cells were then grown for another 24 hours, at which time dihydrotesterone, testosterone, estradiol, progesterone, and dexamethasone (Steraloids Inc., Wilton, NH.) were added at a final concentration of 2 nM. Cells were grown for another 24 hours and RNA was then harvested as previously described and PSM expression analyzed by ribonuclease protection analysis.

Experimental Results

Immunohistochemical Detection of PSM: Using the 7E11-C5.3 anti-PSM monoclonal antibody, PSM expression is clearly detectable in the LNCaP prostate cancer cell line, but not in the PC-3 and DU-145 cell lines (Figures 17A-17C). All normal and malignant prostatic tissues analyzed stained positively for PSM expression.

In-Vitro Transcription/Translation of PSM Antigen: As shown in Figure 18, coupled in-vitro transcription/ translation of the 2.65 kb full-length PSM cDNA yields an 84 kDa protein species in agreement with the expected protein product from the 750 amino acid PSM open reading frame. Following post-translational

modification using pancreatic canine microsomes were obtained a 100 kDa glycosylated protein species consistent with the mature, native PSM antigen.

Detection of PSM Antigen in LNCaP Cell Membranes and Transfected PC-3 Cells: PC-3 cells transfected with the full length PSM cDNA in the pREP7 expression vector were assayed for expression of SM mRNA by Northern analysis. A clone with high PSM mRNA expression was selected for PSM antigen analysis by Western blotting using the 7E11-C5.3 antibody. In Figure 19, the 100 kDa PSM antigen is well expressed in LNCaP cell lysate and membrane fractions, as well as in PSM-transfected PC-3 cells but not in native PC-3 cells. This detectable expression in the transfected PC-3 cells proves that the previously cloned 2.65 kb PSM cDNA encodes the antigen recognized by the 7E11-C5.3 anti-prostate monoclonal antibody.

PSM mRNA Expression: Expression of PSM mRNA in normal human tissues was analyzed using ribonuclease protection assays. Tissue expression of PSM appears predominantly within the prostate, with very low levels of expression detectable in human brain and salivary gland (Figure 20). No detectable PSM mRNA expression was evident in non-prostatic human tissues when analyzed by Northern analysis. On occasion it is noted that detectable PSM expression in normal human small intestine tissue, however this mRNA expression is variable depending upon the specific riboprobe used. All samples of normal human prostate and human prostatic adenocarcinoma assayed have revealed clearly detectable PSM expression, whereas generally decreased or absent expression of PSM in tissues exhibiting benign hyperplasia (Figure 21). In human LNCaP tumors grown both orthotopically and subcutaneously in nude mice abundant PSM expression with or without the use of

matrigel, which is required for the growth of subcutaneously implanted LNCaP cells was detected (Figure 21). PSM mRNA expression is distinctly modulated by the presence of steroids in physiologic doses (Figure 22). DHT downregulated expression by 8-10 fold after 24 hours and testosterone diminished PSM expression by 3-4 fold. Estradiol and progesterone also downregulated PSM expression in LNCaP cells, perhaps as a result of binding to the mutated androgen receptor known to exist in the LNCaP cell. Overall, PSM expression is highest in the untreated LNCaP cells grown in steroid-depleted media, a situation that simulates the hormone-deprived (castrate) state *in-vivo*. This experiment was repeated at steroid dosages ranging from 2-200 nM and at time points from 6 hours to 7 days with similar results; maximal downregulation of PSM mRNA was seen with DHT at 24 hours at doses of 2-20 nM.

Experimental Discussion

Previous research has provided two valuable prostatic bio-markers, PAP and PSA, both of which have had a significant impact on the diagnosis, treatment, and management of prostate malignancies. The present work describing the preliminary characterization of the prostate-specific membrane antigen (PSM) reveals it to be a gene with many interesting features. PSM is almost entirely prostate-specific as are PAP and PSA, and as such may enable further delineation of the unique functions and behavior of the prostate. The predicted sequence of the PSM protein (30) and its presence in the LNCaP cell membrane as determined by Western blotting and immunohistochemistry, indicate that it is an integral membrane protein. Thus, PSM provides an attractive cell surface epitope for antibody-directed diagnostic imaging and cytotoxic

targeting modalities . The ability to synthesize the PSM antigen *in-vitro* and to produce tumor xenografts maintaining high levels of PSM expression provides us with a convenient and attractive model system to further study and characterize the regulation and modulation of PSM expression. Also, the high level of PSM expression in the LNCaP cells provides an excellent *in-vitro* model system. Since PSM expression is hormonally-responsive to steroids and may be highly expressed in hormone-refractory disease. The detection of PSM mRNA expression in minute quantities in brain, salivary gland, and small intestine warrants further investigation, although these tissues were negative for expression of PSM antigen by immunohistochemistry using the 7E11-C5.3 antibody. In all of these tissues, particularly small intestine, mRNA expression using a probe corresponding to a region of the PSM cDNA near the 3' end, whereas expression when using a 5' end PSM probe was not detected. These results may indicate that the PSM mRNA transcript undergoes alternative splicing in different tissues.

Applicants approach is based on prostate tissue specific promotor: enzyme or cytokine chimeras. Promotor specific activation of prodrugs such as non toxic gancyclovir which is converted to a toxic metabolite by herpes simplex thymidine kinase or the prodrug 4-(bis(2chloroethyl)amino)benzoyl-1-glutamic acid to the benzoic acid mustard alkylating agent by the pseudomonas carboxy peptidase G2 was examined. As these drugs are activated by the enzyme (chimera) specifically in the tumor the active drug is released only locally in the tumor environment, destroying the surrounding tumor cells. Promotor specific activation of cytokines such as IL-12, IL-2 or GM-CSF for activation and specific antitumor vaccination is examined. Lastly the tissue specific promotor

activation of cellular death genes may also prove to be useful in this area.

Gene Therapy Chimeras: The establishment of "chimeric DNA" for gene therapy requires the joining of different segments of DNA together to make a new DNA that has characteristics of both precursor DNA species involved in the linkage. In this proposal the two pieces being linked involve different functional aspects of DNA, the promotor region which allows for the reading of the DNA for the formation of mRNA will provide specificity and the DNA sequence coding for the mRNA will provide for therapeutic functional DNA.

DNA-Specified Enzyme or Cytokine mRNA: When effective, antitumor drugs can cause the regression of very large amounts of tumor. The main requirements for antitumor drug activity is the requirement to achieve both a long enough time (t) and high enough concentration (c) (cxt) of exposure of the tumor to the toxic drug to assure sufficient cell damage for cell death to occur. The drug also must be "active" and the toxicity for the tumor greater than for the hosts normal cells. The availability of the drug to the tumor depends on tumor blood flow and the drugs diffusion ability. Blood flow to the tumor does not provide for selectivity as blood flow to many normal tissues is often as great or greater than that to the tumor. The majority of chemotherapeutic cytotoxic drugs are often as toxic to normal tissue as to tumor tissue. Dividing cells are often more sensitive than non-dividing normal cells, but in many slow growing solid tumors such as prostatic cancer this does not provide for antitumor specificity.

Previously a means to increase tumor specificity of antitumor drugs was to utilize tumor associated enzymes to activate nontoxic prodrugs to cytotoxic agents. A

problem with this approach was that most of the enzymes found in tumors were not totally specific in their activity and similar substrate active enzymes or the same enzyme at only slightly lower amounts was found in other tissue and thus normal tissues were still at risk for damage.

To provide absolute specificity and unique activity, viral, bacterial and fungal enzymes which have unique specificity for selected prodrugs were found which were not present in human or other animal cells. Attempts to utilize enzymes such as herpes simplex thymidine kinase, bacterial cytosine deaminase and carboxypeptidase G-2 were linked to antibody targeting systems with modest success. Unfortunately, antibody targeted enzymes limit the number of enzymes available per cell. Also, most antibodies do not have a high tumor target to normal tissue ratio thus normal tissues are still exposed reducing the specificity of these unique enzymes. Antibodies are large molecules that have poor diffusion properties and the addition of the enzymes molecular weight further reduces the antibodies diffusion.

Gene therapy could produce the best desired result if it could achieve the specific expression of a protein in the tumor and not normal tissue in order that a high local concentration of the enzyme be available for the production in the tumor environment of active drug.

Cytokines:

Results demonstrated that tumors such as the bladder and prostate were not immunogenic, that is the administration of irradiated tumor cells to the animal prior to subsequent administration of non-irradiated tumor cells did not result in a reduction of either the number of tumor cells to produce a tumor nor did it

reduce the growth rate of the tumor. But if the tumor was transfected with a retrovirus and secreted large concentrations of cytokines such as Il-2 then this could act as an antitumor vaccine and could also reduce the growth potential of an already established and growing tumor. IL-2 was the best, GM-CSF also had activity whereas a number of other cytokines were much less active. In clinical studies just using IL-2 for immunostimulation, very large concentrations had to be given which proved to be toxic. The key to the success of the cytokine gene modified tumor cell is that the cytokine is produced at the tumor site locally and is not toxic and that it stimulates immune recognition of the tumor and allows specific and non toxic recognition and destruction of the tumor. The exact mechanisms of how IL-2 production by the tumor cell activates immune recognition is not fully understood, but one explanation is that it bypasses the need for cytokine production by helper T cells and directly stimulates tumor antigen activated cytotoxic CD8 cells. Activation of antigen presenting cells may also occur.

Tissue Promotor-Specific Chimera DNA Activation

Non-Prostatic Tumor Systems:

It has been observed in non-prostatic tumors that the use of promotor specific activation can selectively lead to tissue specific gene expression of the transfected gene. In melanoma the use of the tyrosinase promotor which codes for the enzyme responsible for melanin expression produced over a 50 fold greater expression of the promotor driven reporter gene expression in melanoma cells and not non melanoma cells. Similar specific activation was seen in the melanoma cells transfected when they were growing in mice. In that experiment no non-melanoma or melanocyte cell expressed the tyrosinase drive reporter gene

product. The research group at Welcome Laboratories have cloned and sequenced the promoter region of the gene coding for carcinoembryonic antigen (CEA). CEA is expressed on colon and colon carcinoma cells but specifically on metastatic. A gene chimera was generated which cytosine deaminase. Cytosine deaminase which converts 5 flurorocytosine into 5 fluorouracil and observed a large increase in the ability to selectively kill CEA promotor driven colon tumor cells but not normal liver cells. In vivo they observed that bystander tumor cells which were not transfected with the cytosine deaminase gene were also killed, and that there was no toxicity to the host animal as the large tumors were regressing following treatment. Herpes simplex virus, (HSV), thymidine kinase similarly activates the prodrug gancyclovir to be toxic towards dividing cancer cells and HSV thymidine kinase has been shown to be specifically activatable by tissue specific promoters.

Prostatic Tumor Systems: The therapeutic key to effective cancer therapy is to achieve specificity and spare the patient toxicity. Gene therapy may provide a key part to specificity in that non-essential tissues such as the prostate and prostatic tumors produce tissue specific proteins, such as acid phosphatase (PAP), prostate specific antigen (PSA), and a gene which was cloned, prostate-specific membrane antigen (PSM). Tissues such as the prostate contain selected tissue specific transcription factors which are responsible for binding to the promoter region of the DNA of these tissue specific mRNA. The promoter for PSA has been cloned. Usually patients who are being treated for metastatic prostatic cancer have been put on androgen deprivation therapy which dramatically reduces the expression of mRNA for PSA. PSM on the other hand increases in expression with hormone

deprivation which means it would be even more intensely expressed on patients being treated with hormone therapy.

EXAMPLE 3:

CLONING AND CHARACTERIZATION OF THE PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSM) PROMOTER.

The expression and regulation of the PSM gene is complex. By immunostaining, PSM antigen was found to be expressed brilliantly in metastasized tumor, and in organ confined tumor, less so in normal prostatic tissue and more heterogenous in BPH. PSM is strongly expressed in both anaplastic and hormone refractory tumors. PSM mRNA has been shown to be down regulated by androgen. Expression of PSM RNA is also modulated by a host of cytokines and growth factors. Knowledge of the regulation of PSM expression should aid in such diagnostic and therapeutic strategies as imunoscintigraphic imaging of prostate cancer and prostate-specific promoter-driven gene therapy.

Sequencing of a 3 kb genomic DNA clone revealed that two stretches of about 300 B.P. (-260 to -600; and -1325 to -1625) have substantial homology (79-87%) to known genes. The promoter lacks a GC rich region, nor does it have a consensus TATA box. However, it contains a TA-rich region from position -35 to -65.

Several consensus recognition sites for general transcription factors such as AP1, AP2, NFkB, GRE and E2-RE were identified. Chimeric constructs containing fragments of the upstream region of the PSM gene fused to a promoterless chloramphenicol acetyl transferase gene were transfected into, and transiently expressed

in LNCaP, PC-3, and SW620 (a colonic cell line). With an additional SV40 enhancer, sequence from -565 to +76 exhibited promoter activity in LNCaP but not in PC-3 nor in SW620.

Materials and Methods

Cell Lines. LNCaP and PC-3 prostatic carcinoma cell lines (American Type Culture Collection) were cultured in RPMI and MEM respectively, supplemented with 5% fetal calf serum at 37°C and 5% CO_2. SW620, a colonic cell line.

Polymerase Chain Reaction. The reaction was performed in a 50 l volume with a final concentration of the following reagents: 16.6 mM NH_4SO_4, 67 mM Tris-HCl pH 8.8, acetylated BSA 0.2 mg/ml, 2mM $MgCl_2$, 250μM dNTPs, 10 mM ß-mercaptoethanol, and 1 U of the 111 Taq polymerase (Boehringer Mannhiem, CA). A total of 25 cycles were completed with the following profile: cycle 1, 94°C 4 min.; cycle 2 through 25, 94°C 1 min, 60°C 1 min, 72°C 1 min. The final reaction was extended for 10 min at 72°C. Aliquots of the reaction were electrophoresed on 1 % agarose gels in 1X Tris-acetate-EDTA buffer.

Cloning of PSM promoter. A bacteriophage P1 library of human fibroblast genomic DNA (Genomic Sysytems, Inc., St. Louis, MI), was screened using a PCR method of Pierce et al. Primers located at the 5' end of PSM cDNA were used:5'-CTCAAAAGGGGCCGGATTTCC-3' and 5'CTCTCAATCTCACTAATGCCTC-3'. A positive clone, p683, was digested with Xhol restriction enzyme. Southern analysis of the restricted fragments using a DNA probe from the extreme 5' to the Ava-1 site of PSM cDNA confirmed that a 3Kb fragment contains the 5'

regulatory sequence of the PSM gene. The 3 kb XhoI fragment was subcloned into pKSBluescrpt vectors and sequenced using the dideoxy method.

Functional Assay of PSM Promoter. Chloramphenicol Acetyl Transferase, (CAT) gene plasmids were constructed from the SmaI-HindIII fragments or subfragements (using either restriction enzyme subfragments or PCR) by insertion into promoterless pCAT basic or pCAT-enhancer vectors (Promega). pCAT-constructs were cotransfected with pSVßgal plasmid (5 µg of each plasmid) into cell lines in duplicates, using a calcium phosphate method (Gibco-BRL, Gaithersburg, MD). The transfected cells were harvested 72 hours later and assayed (15µg of lysate) for CAT activity using the LSC method and for ßgal activity (Promega). CAT activities were standardized by comparision to that of the ßgal activities.

Results

Sequence of the 5' end of the PSM gene.

The DNA sequence of the 3 kb XhoI fragment of p683 which includes 3017 bp of DNA from the RNA start site was determined. (Figure 15) The sequence from the XhoI fragment displayed a remarkable arrays of elements and motifs which are characteristic of eukaryotic promoters and regulatory regions found in other genes (Figure 16).

Functional Analysis of upstream PSM genomic elements for promoter activity.

Various pCAT-PSM promoter constructs were tested for promoter activities in two prostatic cell lines: LNCaP, PC-3 and a colonic SW620 (Figure 17). Induction of CAT activity was neither observed in p1070-CAT which

contained a 1070 bp PSM 5' promoter fragment, nor in p676-CAT which contained a 641 bp PSM 5' promoter fragment. However, with an additional SV-40 enhancer, sequence from -641 to -1 (p676-CATE) exhibited promoter activity in LNCaP but not in PC-3 nor in SW620.

Therefore, a LNCaP specific promoter fragment from -641 to -1 has been isolated which can be used in PSM promoter-driven gene therapy.

EXAMPLE 4:

ALTERNATIVELY SPLICED VARIANTS OF PROSTATE SPECIFIC MEMBRANE ANTIGEN RNA: RATIO OF EXPRESSION AS A POTENTIAL MEASUREMENT OF PROGRESSION

MATERIALS AND METHODS

Cell Lines. LNCaP and PC-3 prostatic carcinoma cell lines were cultured in RPMI and MEM respectively, supplemented with 5% fetal calf serum at 37°C and 5% CO_2.

Primary tissues. Primary prostatic tissues were obtained from MSKCC's in-house tumor procurement service. Gross specimen were pathologically staged by MSKCC's pathology service.

RNA Isolation. Total RNA was isolated by a modified guanidinium thiocynate/phenol/chloroform method using a RNAzol B kit (Tel-Test, Friendswood, TX). RNA was stored in diethyl pyrocarbonate-treated water at -80°C. RNA was quantified using spectrophometric absorption at 260nm.

cDNA synthesis. Two different batches of normal prostate mRNAs obtained from trauma-dead males

(Clontech, Palo Alto, CA) were denatured at 70°C for 10 min., then reverse transcribed into cDNA using random hexamers and Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) at 50°C for 30 min. followed by a 94°C incubation for 5 min.

Polymerase Chain Reaction. Oligonucleotide primers (5'-CTCAAAAGGGGCCGGATTTCC-3' and 5'-AGGCTACTTCACTCAAAG-3'), specific for the 5' and 3' ends of PSM cDNA were designed to span the cDNA sequence. The reaction was performed in a 50 µl volume with a final concentration of the following reagents: 16.6 mM NH_4SO_4, 67 mM Tris-HCl pH 8.8, acetylated BSA 0.2 mg/ml, 2mM $MgCl_2$, 250µM dNTPs, 10 mM ß-mercaptoethanol, and 1 U of rTth polymerase (Perkin Elmer, Norwalk, CT). A total of 25 cycles were completed with the following profile: cycle 1, 94°C 4 min.; cycle 2 through 25, 94°C 1 min, 60°C 1 min, 72°C 1 min. The final reaction was extended for 10 min at 72°C. Aliquots of the reaction were electrophoresed on 1 % agarose gels in 1X Tris-acetate-EDTA buffer.

Cloning of PCR products. PCR products were cloned by the TA cloning method into pCRII vector using a kit from Invitrogen (San Diego, CA). Ligation mixture were transformed into competent *Escherichia coli* Inv5α.

Sequencing. Sequencing was done by the dideoxy method using a sequenase kit from US Biochemical (Cleveland, OH). Sequencing products were electrophoresed on a 5% polyacrylamide/7M urea gel at 52°C.

RNase Protection Assays. Full length PSM cDNA clone was digested with NgoM 1 and Nhe1. A 350 b.p. fragment was isolated and subcloned into pSPORT1 vector (GIBCO-BRL, Gaithersburg, MD). The resultant plasmid, pSP350,

was linearized, and the insert was transcribed by SP6 RNA polymerase to yield antisense probe of 395 nucleotide long, of which 355 nucleotides and/or 210 nucleotides should be protected from RNAse digestion by PSM RNA respectively. Total celluar RNA (20 μg) from different tissues were hybridized to the aforementioned antisense RNA probe. Assays were performed as described. tRNA was used as negative control. RPAs for LNCaP and PC-3 were repeated.

RESULTS

RT-PCR of mRNA from normal prostatic tissue. Two independent RT-PCR of mRNA from normal prostates were performed as described in *Materials and Methods*. Subsequent cloning and sequencing of the PCR products revealed the presence of an alternatively spliced variant, PSM'. PSM' has a shorter cDNA (2387 nucleotides) than PSM (2653 nucleotides). The results of the sequence analysis are shown in Figure 18. The cDNAs are identical except for a 266 nucleotide region near the 5' end of PSM cDNA (nucleotide 114 to 380) that is absent in PSM' cDNA. Two independent repetitions of RT-PCR of different mRNA samples yielded identical results.

RNase Protection Assays. An RNA probe complementary to PSM RNA and spanning the 3' splice junction of PSM' RNA was used to measure relative expression of PSM and PSM' mRNAs (Figure 19). With this probe, both PSM and PSM' RNAs in LNCaP cells was detected and the predominant form was PSM. Neither PSM nor PSM' RNA was detected in PC-3 cells, in agreement with previous Northern and Western blot data. Figure 20 showed the presence of both splice variants in human primary prostatic tissues. In primary prostatic tumor, PSM is the dominant form. In contrast, normal prostate expressed more PSM' than PSM. BPH samples showed about equal

expression of both variants.

Tumor Index. The relative expression of PSM and PSM' (Figure 36) was quantified by densitometry and expressed as a tumor index (Figure 21). LNCaP has an index ranging from 9-11; CaP from 3-6; BPH from 0.75 to 1.6; normal prostate has values from 0.075 to 0.45.

DISCUSSION

Sequencing data of PCR products derived from human normal prostatic mRNA with 5' and 3' end PSM oligonucleotide primers revealed a second splice variant, PSM', in addition to the previously described PSM cDNA.

PSM is a 750 a.a. protein with a calculated molecular weight of 84,330. PSM was hypothesized to be a type II integral membrane protein. A classic type II membrane protein is the transferrin receptor and indeed PSM has a region that has modest homology with the transferrin receptor. Analysis of the PSM amino acid sequence by either the methods of Rao and Argos or Eisenburg *et. al.* strongly predicted one transmembrane helix in the region from a.a.#20 to #43. Both programs found other regions that could be membrane associated but were not considered likely candidates for being transmembrane regions.

PSM' antigen, on the other hand, is a 693 a.a. protein as deduced from its mRNA sequence with a molecular weight of 78,000. PSM' antigen lacks the first 57 amino acids present in PSM antigen (Figure 18). It is likely that PSM' antigen is cytosolic.

The function of PSM and PSM' are probably different. The cellular location of PSM antigen suggests that it may interact with either extra- or intra- cellular

ligand(s) or both; while that of PSM' implies that PSM' can only react with cytosolic ligand(s). Furthermore, PSM antigen has 3 potential phosphorylation sites on its cytosolic domain. These sites are absent in PSM' antigen. On the other hand, PSM' antigen has 25 potential phosphorylation sites, 10 N-myristoylation sites and 9 N-glycosylation sites. For PSM antigen, all of these potential sites would be on the extracellular surface. The modifications of these sites for these homologous proteins would be different depending on their cellular locations. Consequently, the function(s) of each form would depend on how they are modified.

The relative differences in expression of PSM and PSM' by RNase protection assays was analyzed. Results of expression of PSM and PSM' in primary prostatic tissues strongly suggested a relationship between the relative expression of these variants and the status of the cell: either normal or cancerous. While it is noted here that the sample size of the study is small (Figures 20 and 21), the consistency of the trend is evident. The samples used were gross specimens from patients. The results may have been even more dramatic if specimens that were pure in content of CaP, BPH or normal had been used. Nevertheless, in these specimens, it is clear that there is a relative increase of PSM over PSM' mRNA in the change from normal to CaP. The Tumor Index (Figure 21) could be useful in measuring the pathologic state of a given sample. It is also possible that the change in expression of PSM over PSM' may be a reason for tumor progression. A more differentiated tumor state may be restored by PSM' either by transfection or by the use of differentiation agents.

EXAMPLE 5:

ENHANCED DETECTION OF PROSTATIC HEMATOGENOUS MICRO-METASTASES WITH PSM PRIMERS AS COMPARED TO PSA PRIMERS USING A SENSITIVE NESTED REVERSE TRANSCRIPTASE-PCR ASSAY.

77 randomly selected samples were analyzed from patients with prostate cancer and reveals that PSM and PSA primers detected circulating prostate cells in 48 (62.3%) and 7 (9.1%) patients, respectively. In treated stage D disease patients, PSM primers detected cells in 16 of 24 (66.7%), while PSA primers detected cells in 6 of 24 patients (25%). In hormone-refractory prostate cancer (stage D3), 6 of 7 patients were positive with both PSA and PSM primers. All six of these patients died within 2-6 months of their assay, despite aggressive cytotoxic chemotherapy, in contrast to the single patient that tested negatively in this group and is alive 15 months after his assay, suggesting that PSA-PCR positivity may serve as a predictor of early mortality. In post-radical prostatectomy patients with negative serum PSA values, PSM primers detected metastases in 21 of 31 patients (67.7%), while PSA primers detected cells in only 1 of 33 (3.0%), indicating that micrometastatic spread may be a relatively early event in prostate cancer. The analysis of 40 individuals without known prostate cancer provides evidence that this assay is highly specific and suggests that PSM expression may predict the development of cancer in patients without clinically apparent prostate cancer. Using PSM primers, micrometastases were detected in 4 of 40 controls, two of whom had known BPH by prostate biopsy and were later found to have previously undetected prostate cancer following repeat prostate biopsy performed for a rising serum PSA value. These results

show the clinical significance of detection of hematogenous micrometastatic prostate cells using PSM primers and potential applications of this molecular assay.

EXAMPLE 6:

MODULATION OF PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSM) EXPRESSION IN VITRO BY CYTOKINES AND GROWTH FACTORS.

The effectiveness of CYT-356 imaging is enhanced by manipulating expression of PSM. PSM mRNA expression is downregulated by steroids. This is consistent with the clinical observations that PSM is strongly expressed in both anaplastic and hormone refractory lesions. In contrast, PSA expression is decreased following hormone withdrawal. In hormone refractory disease, it is believed that tumor cells may produce both growth factors and receptors, thus establishing an autocrine loop that permits the cells to overcome normal growth constraints. Many prostate tumor epithelial cells express both TGFα and its receptor, epidermal growth factor receptor. Results indicate that the effects of TGFα and other selected growth factors and cytokines on the expression of PSM in-vitro, in the human prostatic carcinoma cell line LNCaP.

$2x10^6$ LNCaP cells growing in androgen-depleted media were treated for 24 to 72 hours with EGF, TGFα, TNFß or TNFα in concentrations ranging from 0.1 ng/ml to 100 ng/ml. Total RNA was extracted from the cells and PSM mRNA expression was quantitated by Northern blot analysis and laser densitometry. Both b-FGF and TGFα yielded a dose-dependent 10-fold upregulation of PSM expression, and EGF a 5-fold upregulation, compared to untreated LNCaP. In contrast, other groups have shown a marked downregulation in PSA expression induced by

these growth factors in this same in-vitro model. TNFα, which is cytotoxic to LNCaP cells, and TNFß downregulated PSM expression 8-fold in androgen depleted LNCaP cells.

TGFα is mitogenic for aggressive prostate cancer cells. There are multiple forms of PSM and only the membrane form is found in association with tumor progression. The ability to manipulate PSM expression by treatment with cytokines and growth factors may enhance the efficacy of Cytogen 356 imaging, and therapeutic targeting of prostatic metastases.

EXAMPLE 7:

NEOADJUVANT ANDROGEN-DEPRIVATION THERAPY (ADT) PRIOR TO RADICAL PROSTATECTOMY RESULTS IN A SIGNIFICANTLY DECREASED INCIDENCE OF RESIDUAL MICROMETASTATIC DISEASE AS DETECTED BY NESTED RT-PCT WITH PRIMERS.

Radical prostatectomy for clinically localized prostate cancer is considered by many the "gold standard" treatment. Advances over the past decade have served to decrease morbidity dramatically. Improvements intended to assist clinicians in better staging patients preoperatively have been developed, however the incidence of extra-prostatic spread still exceeds 50%, as reported in numerous studies. A phase III prospective randomized clinical study designed to compare the effects of ADT for 3 months in patients undergoing radical prostatectomy with similarly matched controls receiving surgery alone was conducted. The previously completed phase II study revealed a 10% margin positive rate in the ADT group (N=69) as compared to a 33% positive rate (N=72) in the surgery alone group.

Patients who have completed the phase III study were analyzed to determine if there are any differences between the two groups with respect to residual micrometastatic disease. A positive PCR result in a post-prostatectomy patient identifies viable metastatic cells in the circulation.

Nested RT-PCR was performed with PSM primers on 12 patients from the ADT group and on 10 patients from the control group. Micrometastatic cells were detected in 9/10 patients (90%) in the control group, as compared to only 2/12 (16.7%) in the ADT group. In the ADT group, 1 of 7 patients with organ-confined disease tested positively, as compared to 3 of 3 patients in the control group. In patients with extra-prostatic disease, 1 of 5 were positive in the ADT group, as compared to 6 of 7 in the control group. These results indicate that a significantly higher number of patients may be rendered tumor-free, and potentially "cured" by the use of neoadjuvant ADT.

EXAMPLE 8:

SENSITIVE NESTED RT-PCR DETECTION OF CIRCULATION PROSTATIC TUMOR CELLS - COMPARISON OF PSM AND PSA-BASED ASSAYS

Despite the improved and expanded arsenal of modalities available to clinician today, including sensitive serum PSA assays, CT scan, transrectal ultrasonography, endorectal co.I MRI, etc., many patients are still found to have metastatic disease at the time of pelvic lymph node dissection and radical prostatectomy. A highly sensitive reverse transcription PCR assay capable of detecting occult hematogenous micrometastatic prostatic cells that would otherwise go undetected by presently available staging modalities

was developed. This assay is a modification of similar PCR assays performed in patients with prostate cancer and other malignancies. The assay employs PCR primers derived from the cDNA sequences of prostate-specific antigen[6] and the prostate-specific membrane antigen recently cloned and sequenced.

Materials and Methods

Cells and Reagents. LNCaP and MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD.). Details regarding the establishment and characteristics of these cell. Cells grown in RPMI 1640 medium and supplemented with L-glutamine, nonessential amino acids, and 5% fetal calf serum (Gibco-BRL, Gaithersburg, MD.) In a 5% CO_2 incubator at 37°C. All cell media was obtained from the MSKCC Media Preparation Facility. Routine chemical reagents were of the highest grade possible and were obtained from Sigma Chemical Company (St. Louis, MO).

Patient Blood Specimens. All blood specimens used in this study were from patients seen in the outpatient offices of urologists on staff at MSKCC. Two anti-coagulated tubes per patient were obtained at the time of their regularly scheduled blood draws. Specimens were obtained with informed consent of each patient , as per a protocol approved by the MSKCC Institutional Review Board. Samples were promptly brought to the laboratory for immediate processing. Seventy-seven specimens from patients with prostate cancer were randomly selected and delivered to the laboratory "blinded" along with samples from negative controls for processing. These included 24 patients with stage D disease (3 with D_0, 3 with D^1, 11 with D^2, and 7 with D^3), 31 patients who had previously undergone radical prostatectomy and had undetectable postoperative serum

PSA levels (18 with pT2 lesions, 11 with pT3, and 2 pT4), 2 patients with locally recurrent disease following radical prostatectomy, 4 patients who had received either external beam radiation therapy or interstitial I^{125} implants, 10 patients with untreated clinical stage T1-T2 disease, and 6 patients with clinical stage T3 disease on anti-androgen therapy. The forty blood specimens used as negative controls were from 10 health males, 9 males with biopsy-proven BPH and elevated serum PSA levels, 7 healthy females, 4 male patients with renal cell carcinoma, 2 patients with prostatic intraepithelial neoplasia (PIN), 2 patients with transitional cell carcinoma of the bladder and a pathologically normal prostate, 1 patient with acute prostatitis, 1 patient with acute promyelocytic leukemia, 1 patient with testicular cancer, 1 female patient with renal cell carcinoma, 1 patient with lung cancer, and 1 patient with a cyst of the testicle.

Blood Sample Processing/RNA Extraction. 4 ml of whole anticoagulated venous blood was mixed with 3 ml of ice cold PBS and then carefully layered atop 8 ml of Ficoll (Pharmacia, Uppsala, Sweden) in a 14-ml polystyrene tube. Tubes were centrifuged at 200 x g for 30 min. at 4°C. The buffy coat layer (approx. 1 ml.) was carefully removed and rediluted to 50 ml with ice cold PBS in a 50 ml polypropylene tube. This tube was then centrifuged at 2000 x g for 30 min. at 4°C. The supernatant was carefully decanted and the pellet was allowed to drip dry. One ml of RNazol B was then added to the pellet and total RNA was isolated as per manufacturers directions (Cinna/Biotecx, Houston, TX.) RNA concentrations and purity were determined by UV spectroscopy on a Beckman DU 640 spectrophotometer and by gel analysis.

Determination of PCR Sensitivity. RNA was isolated from LNCaP cells and from mixtures of LNCaP and MCF-7 cells at fixed ratios (i.e. 1:100, 1:1,000, etc.) using RNAzol B. Nested PCR was then performed as described below with both PSA and PSM primers in order to determine the limit of detection for the assay. LNCaP:MCF-7 (1:100,000) cDNA was diluted with distilled water to obtain concentrations of 1:1,000,000. The human breast cancer cell line MCF-7 was chosen because they had previously been tested by us and shown not to express either PSM nor PSA by both immunohistochemistry and conventional and nested PCR.

Polymerase Chain Reaction. The PSA outer primer sequences are nucleotides 494-513 (sense) in exon 4 and nucleotides 960-979 (anti-sense) in exon 5 of the PSA cDNA. These primers yield a 486 bp PCR product from PSA CDNA that can be distinguished from a product synthesized from possible contaminating genomic DNA.

PSA-494 5'-TAC CCA CTG CAT CAG GAA CA-3'

PSA-960 5'-CCT TGA AGC ACA CCA TTA CA-3'

The PSA inner upstream primer begins at nucleotide 559 and the downstream primer at nucleotide 894 to yield a 355 bp PCR product.

PSA-559 5'-ACA CAG GCC AGG TAT TTC AG-3'

PSA-894 5'-GTC CAG CGT CCA GCA CAC AG-3'

All primers were synthesized by the MSKCC Microchemistry Core Facility. 5μg of total RNA was reverse-transcribed into cDNA using random hexamer primers (Gibco-BRL) and Superscript II reverse transcriptase (Gibco-BRL) according to the manufacturers recommendations. 1μl of this CDNA served as the starting template for the outer primer PCR reaction. The 20μl PCR mix included: 0.5U Taq polymerase (Promega) Promega reaction buffer, 1.5mM $MgCl_2$, 200μM dNTPs, and 1.0μM of each primer. This mix was then transferred to a Perkin Elmer 9600 DNA thermal

cycler and incubated for 25 cycles. The PCR profile was as follows: 94°C x 15 sec., 60°C x 15 sec., and 72°C for 45 sec. After 25 cycles, samples were placed on ice, and 1µl of this reaction mix served as the template for another 25 cycles using the inner primers. The first set of tubes were returned to the thermal cycler for 25 additional cycles. The PSM outer upstream primer sequences are nucleotides 1368-1390 and the downstream primers are nucleotides 1995-2015, yielding a 67 bp PCR product.

PSM-1368 5'-CAG ATA TGT CAT TCT GGG AGG TC-3'
PSM-2015 5'-AAC ACC ATC CCT CCT CGA ACC-3'

The PSM inner upstream primer span nucleotides 1689-1713 and the downstream primer span nucleotides 1899-1923, yielding a 234 bp PCR product.

PSM-1689 5'-CCT AAC AAA AGA GCT GAA AAG CCC-3'
PSM-1923 5'-ACT GTG ATA CAG TGG ATA GCC GCT-3'

2µl of cDNA was used as the starting DNA template in the PCR assay. The 50µl PCR mix included: 1U Taq polymerase (Boehringer Mannheim), 250µM cNTPs, 10mM ß-mercaptoethanol, 2mM $MgCl_2$, and 5µl of a 10x buffer mix containing: 166mM NH_4SO_4, 670mM Tris pH 8.8, and 2mg/ml of acetylated BSA. PCR was carried out in a Perkin Elmer 480 DNA thermal cycler with the following parameters: 94°C x 4 minutes for 1 cycle, 94°C x 30 sec., 58°C x 1 minute, and 72°C x 1 minute for 25 cycles, followed by 72°C x 10 minutes. Samples were then iced and 2.5µl of this reaction mix was used as the template for another 25 cycles with a new reaction mix containing the inner PSM primers. cDNA quality was verified by performing control reactions using primers derived from the ß-2-microglobulin gene sequence[10] a ubiquitous housekeeping gene. These primers span exons 2-4 and generate a 620 bp PCR product. The sequences for these primers are:

ß-2 (exon 2) 5'-AGC AGA GAA TGG AAA GTC AAA-3'

ß-2 (exon 4) 5'-TGT TGA TGT TGG ATA AGA GAA-3'

The entire PSA mix and 7-10µl of each PSM reaction mix were run on 1.5-2% agarose gels, stained with ethidium bromide and photographed in an Eage Eye Video Imaging System (Statagene, Torrey Pines, CA.). Assays were repeated at least twice to verify results.

Cloning and Sequencing of PCR Products. PCR products were cloned into the pCR II plasmid vector using the TA cloning system (Invitrogen). These plasmids were transformed into competent E. coli cells using standard methods[11] and plasmid DNA was isolated using Magic Minipreps (Promega) and screened by restriction analysis. Double-stranded TA clones were then sequenced by the dideoxy method using ^{35}S-cCTP (NEN) and Sequenase (U.S. Biochemical). Sequencing products were then analyzed on 6% polyacrilamide/7M urea gels, which were fixed, dried, and autoradiographed as described.

Southern Analysis. PCR products were transferred from ethidium-stained agarose gels to Nytran nylon membranes (Schletcher and Schuell) by pressure blotting with a Posi-blotter (Stratagene) according to the manufacturer's instructions. DNA was cross-linked to the membrane using a UV Stratalinker (Stratagene). Blots were pre-hybridized at 65°C for 2 hours and subsequently hybridized with denatured ^{32}P-labeled, random-primed cDNA probes (either PSA or PSM).[6,7] Blots were washed twice in 1x SSC/0.5% SDS at 42°C and twice in 0.1x SSC/0.1% SDS at 50°C for 20 minutes each. Membranes were air-dried and autoradiographed for 1-3 hours at room temperature with Hyperfilm MP (Amersham).

Results

PSA and PSM Nested PCR Assays: The application of nested PCR increased the level of detection from an

average of 1:10,000 using outer primers alone, to better than 1:1,000,000. Dilution curves demonstrating this added sensitivity are shown for PSA and PSM-PCR in Figures 1 and 2 respectively. Figure 1 shows that the 486 bp product of the PSA outer primer set is clearly detectable with ethidium staining to 1:10,000 dilutions, whereas the PSA inner primer 355 bp product is clearly detectable in all dilutions shown. In Figure 2 the PSM outer primer 647 bp product is also clearly detectable in dilutions to only 1:10,000 with conventional PCR, in contrast to the PSM inner nested PCR 234 bp product which is detected in dilutions as low as 1:1,000,000. Southern blotting was performed on all controls and most of the patient samples in order to confirm specificity. Southern blots of the respective dilution curves confirmed the primer specificities but did not reveal any significantly increased sensitivity.

PCR in Negative Controls: Nested PSA and PSM PCR was performed on 40 samples from patients and volunteers as described in the methods and materials section. Figure 48 reveals results from 4 representative negative control specimens, in addition to a positive control. Each specimen in the study was also assayed with the ß-2-microglobulin control, as shown in the figure, in order to verify RNA integrity. Negative results were obtained on 39 of these samples using the PSA primers, however PSM nested PCR yielded 4 positive results. Two of these "false positives" represented patients with elevated serum PSA values and an enlarged prostate who underwent a transrectal prostate biopsy revealing stromal and fibromuscular hyperplasia. In both of these patients the serum PSA level continued to rise and a repeat prostate biopsy performed at a later date revealed prostate cancer. One patient who presented to the clinic with a testicular cyst was noted to have a

positive PSM nested PCR result which has been unable to explain. Unfortunately, this patient never returned for follow up, and thus have not been able to obtain another blood sample to repeat this assay. Positive result were obtained with both PSA and PSM primers in a 61 year old male patient with renal cell carcinoma. This patient has a normal serum PSA level and a normal digital rectal examination. Overall, if the two patients were excluded in whom a positive PCR, but no other clinical test, accurately predicted the presence of prostate cancer, 36/38 (94.7%) of the negative controls were negative with PSM primers, and 39/40 (97.5%) were negative using PSA primers.

Patient Samples: In a "blinded" fashion, in which the laboratory staff were unaware of the nature of each specimen, 117 samples from 77 patients mixed randomly with 40 negative controls were assayed. The patient samples represented a diverse and heterogeneous group as described earlier. Several representative patient samples are displayed in Figure 49, corresponding to positive results from patients with both localized and disseminated disease. Patients 4 and 5, both with stage D prostate cancer exhibit positive results with both the outer and inner primer pairs, indicating a large circulating tumor cell burden, as compared to the other samples. Although the PSM and PSA primers yielded similar sensitivities in LNCaP dilution curves as previously shown, PSM primers detected micrometastases in 62.3% of the patient samples, whereas PSA primers only detected 9.1%. In patients with documented metastatic prostate cancer (stages D_0 - D_3) receiving anti-androgen treatment, PSM primers detected micrometastases in 16/24 (66.7%), whereas PSA primers detected circulating cells in only 6/24 (25%). In the study 6/7 patients with hormone-refractory prostate cancer (stage D_3) were positive. In the

study, PSA primers revealed micrometastatic cells in only 1/15 (6.7%) patients with either pT3 or pT4 (locally-advanced) prostate cancer following radical prostatectomy. PSM primers detected circulating cells in 9/15 (60%) of these patients. Interestingly, circulating cells 13/18 (72.2%) patients with pT2 (organ-confined) prostate cancer following radical prostatectomy using PSM primers was detected. None of these patient samples were positive by PSA-PCR.

Improved and more sensitive method for the detection of minimal, occult micrometastic disease have been reported for a number of malignancies by use of immunohistochemical methods, as well as the polymerase chain reaction. The application of PCR to detect occult hematogenous micrometastases in prostate cancer was first described by Moreno, et al. using conventional PCR with PSA-derived primers.

When human prostate tumors and prostate cancer cells in-vitro were studied by immunohistochemistry and mRNA analysis, PSM appeared to be highly expressed in anaplastic cells, hormone-refractory cells, and bony metastases, in contrast to PSA. If cells capable of hematogenous micrometastasis represent the more aggressive and poorly-differentiated cells, they may express a higher level of PSM per cell as compared to PSA, enhancing their detectibility by RT-PCR.

Nested RT-PCR assays are both sensitive and specific. Results have been reliably reproduced on repeated occasions. Long term testing of both cDNA and RNA stability is presently underway. Both assays are capable of detecting one prostatic cell in at least one million non-prostatic cells of similar size. This confirms the validity of the comparison of PSM vs. PSA primers. Similar levels of PSM expression in both

human prostatic cancer cells in-vivo and LNCaP cells in-vitro resulted. The specificity of the PSM-PCR assay was supported by the finding that two "negative control" patients with positive PSM-PCR results were both subsequently found to have prostate cancer. This suggests an exciting potential application for this technique for use in cancer screening. In contrast to recently published data, significant ability for PSA primers to accurately detect micrometastatic cells in patients with pathologically with pathologically organ-confined prostate cancer, despite the sensitivity of the assay failed to result. Rather a surprisingly high percentage of patients with localized prostate cancer that harbor occult circulating prostate cells following "curative" radical prostatectomy results which suggests that micrometastasis is an early event in prostate cancer.

The application of this powerful new modality to potentially stage and/or follow the response to therapy in patients with prostate cancer certainly merits further investigation. In comparison to molecular detection of occult tumor cells, present clinical modalities for the detection of prostate cancer spread appear inadequate.

Transition of prostate cancer from androgen dependent to androgen independent state is a clinically important step which may be caused or accompanied by genetic changes. Expression of prostate specific membrane antigen (PSM) is most intense in LNCap cells, an androgen dependent prostate carcinoma cell line: and is not detectable in PC-3 nor in DU-145 cells, which are androgen independent prostate carcinoma cell lines. A microsatellite repeat of (TTTTG), (TTTG), has been found in the first intron of the PSM gene. Our hypothesis is that this Microsatellite repeat could be

a cis-acting element in the regulation of PSM expression. A polymeric chain reaction amplifying this repeat was used to look for any gene alteration in several cell lines: LNCap, PC-3, PC-3M, DU-145 as well as in 20 paired normal and early prostatic cancers (p12-4, NO). In addition, immunohistochemistry (IHC) was used to analyze PSM expression in patient samples. By IHC, no detectable expression in DU-145, PC-3, and PC-3M was found, but all tumor expressed PSM. Further sequencing data of the microsatellite repeat confirmed no change in LNCap, and in contrast, an amplification in PC-3 and a gross deletion in DU-145. Alteration of a T segment adjacent to the microsatellite repeat was found in one tumor sample. These results suggest that there is rarely alteration in the intronic microsatellite sequence of the PSM gene in early prostate cancer. The abnormal pattern in the absence of expression suggest genetic instability in the more aggressive tumor lines such as the PC-3, PC-3M and DU-145 cells.

EXAMPLE 9:

CHROMOSOMAL LOCALIZATION OF COSMID CLONES 194 AND 683 BY FLUORESCENCE IN-SITU HYBRIDIZATION:

PSM was initially mapped as being located on chromosome 11p11.2-p13 (Figures 25-27). Further information from the cDNA in-situ hybridizations experiments demonstrated as much hybridization on the q as p arms. Much larger fragments of genomic DNA was obtained as cosmids and two of these of about 60 kilobases each one going 3' and the other 5' both demonstrated binding to chromosome 11 p and q under low stringency. However under higher stringency conditions only the binding at 11q14-q21 remained. This result suggests that there is another gene on 11p that is very similar to PSM because it is so strongly binding to nearly 120 kilobases of genomic DNA (Figure 28).

Purified DNA from cosmid clones 194 and 683 was labelled with biotin dUTP by nick translation. Labelled probes were combined with sheared human DNA and independently hybridized to normal metaphase chromosomes derived from PHA stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dectran sulfate, and 2XSSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresein conjugated avidin. Following signal detection the slides were counterstained with propidium iodide and analyzed. These first experiments resulted in the specific labelling of a group C chromosome on both the long and short arms. This chromosome was believed to be chromosome 11 on the basis of its size and morphology. A second set of experiments were performed in which a chromosome 11 centromere specific probe was

cohybridized with the cosmid clones. These experiments were carried out in 60% formamide in an attempt to eliminate the cross reactive signal which was observed when low stringency hybridizations were done. These experiments resulted in the specific labelling of the centromere and the long arm of chromosome 11. Measurements of 10 specifically labelled chromosomes 11 demonstrated that the cosmid clones are located at a position which is 44% of the distance from the centromere to the telomere of chromosome arm 11q, an area that corresponds to band 14q. A total of 160 metaphase cells were examined with 153 cells exhibiting specific labelling.

Cloning of the 5' upstream and 3' downstream regions of the PSM genomic DNA. A bacteriophage P1 library of human fibroblast genomic DNA (Genomic Systems, St. Louis, MI) was screened using the PCR method of Pierce et. al. Primer pairs located at either the 5' or 3' termini of PSM cDNA were used. Positive cosmid clones were digested with restriction enzymes and confirmed by Southern analysis using probes which were constructed from either the 5' or 3' ends of PSM cDNA. Positive clone p683 contains the 5' region of PSM cDNA and about 60 kb upstream region. Clone -194 contains the 3' terminal of the PSM cDNA and about 60 kb downstream.

EXAMPLE 10: PEPTIDASE ENZYMATIC ACTIVITY

Prostate Specific Membrane Antigen has activity as a carboxypeptidase and acts on both gamma linked or alpha linked amino acids which have acidic amino acids such as glutamate in the carboxy terminus.

Prostate specific membrane antigen is found in high concentration in the seminal plasma. PSM antigen has enzymatic activity with N-acetylaspartylglutamate as a

substrate and enzymatic action results in the release of, N-acetylaspartate and glutamic acid. Because PSM action will release glutamate, and because it is well known that the seminal fluid is highly enriched in its content of glutamic acid, the action of PSM antigen of endogenous protein/peptide substrates may be responsible for generating the glutamic acid present.

It is also uncertain as to the role that seminal plasma glutamic acid plays in fertility functions. It may be that interruption of PSM antigen enzymatic activity may block the generation of glutamate and could impact on seminal plasma glutamic acid levels and its attendant fertility functions. Thus agents which inhibit PSM antigen may prove to be useful in attenuating male fertility.

EXAMPLE 11: **IONOTROPICGLUTAMATE RECEPTORS IN PROSTATE TISSUE**

Prostate Specific Membrane antigen acts on N-acetylaspartylglutamic acid to release glutamate and because a homologous protein has been found in the rat brain which acts on N-acetlyaspartylglutamate to free glutamate and N-acetylaspartate and because these amino acids are considered to function as neurotransmitters, the enzyme is considered to be potentially important in modulating neurotransmitter excitatory amino acid signalling as a neurocarboxypeptidase. This could be important in the prostate as well, because of the neuroendocrine nature of a subpopulation of cells in the prostate which are considered to be important synthesizeing neuropeptide signaling molecules. PSM antigen from the LNCaP cell was isolated and LNCaP cells can be induced to exhibit a "neuron like" phenotype.

Excitatory neurotransmission in the central nervous system (CNS) is mediated predominantly by glutamate receptors. Two types of glutamate receptors have been identified in the human CNS: metabotropic receptors, which serve G-protein coupled second messenger signalling systems, and ionotropic receptors, which serve as ligand gated ion channels. Ionotropic glutamate channels can increase the inward flow of ions such as calcium ions. This can result in the subsequent stimulation of nitric oxide, and nitric oxide modulation of a number of signalling pathways. Nitric oxide has been found to be a major signalling mechanism involved in cell growth and death, response to inflammation, smooth muscle cell contraction etc.

Methods: Detection of glutamate receptor expression was performed using anti-gluR2/3 and anti-gluR4 polyclonal antibodies and antibiotin immunohistochemical techniques in paraffin-embedded human prostate tissues.

Results: Anti-gluR2/3 immunoreactivity was unique to prostatic stroma and was absent in the prostatic epithelial compartment. Strong anti-gluR4 immunoreactivity was observed in the basal cells of the prostate. This implied a differential location and function of glutamate receptors as defined by these antibodies.

Discussion: Distribution of glutamate receptors in the prostate has not been described. Basal cells are considered the precursor cell for the prostatic acinar and neuroendocrine cells of the prostate. Glutamate receptors may provide signalling functions in their interactions with the prostate stroma and acinar cells, and PSM may be involved in that interaction. Thus inhibition or enhancement of PSM activity could serve to modulate activity of the basal cells and prove to be

a valuable aid for controlling basal cell function in the prostate.

The finding of glutamate like receptors in the stroma is of interest because a large part of the prostate volume is due to stromal cells. Current observation have suggested that these stromal cells have a smooth muscle cell phenotype and thus the presence of glutamate receptors may play a role in their biologic function and regulation of differentiation. A most common disease in men is the abnormal benign growth of the prostate termed benign prostatic hyperplasia, BPH.

In areas of BPH a decrease in the level of expression of PSM antigen was observed. If PSM antigen activity is providing an aspect of the signalling for normal stromal function then the abnormal growth seen in BPH may be a response to that decreased activity and agents to restore its function could play a role in the treatment or prevention of BPH.

Altering PSM antigen function may have beneficial actions outside the prostate. In the rat CNS a protein homology to PSM antigen was discovered and provides a rational to consider prostate specific membrane antigen as a neurocarboxypeptidase. Alterations in its function may occur in neurotoxic disorders such as epilepsy, or ALS, alzheimers, and multiple sclerosis.

EXAMPLE 12: IDENTIFICATION OF A MEMBRANE-BOUND PTEROYLPOLYGAMMAGLUTAMYL CARBOXYPEPTIDASE (FOLATE HYDROLASE) THAT IS EXPRESSED IN HUMAN PROSTATIC CARCINOMA

As described PSM functions as a carboxypeptidase to hydrolyze both alpha and gamma peptide linkages with amino acids such as glutamate in the terminal carboxy position. The proximal small intestine (duodenum-strong expression PSM) but not the distal small intestine (ileum-absent PSM) was also very rich in expression of message for prostate specific membrane antigen in RNase protection assays. PSM antigen by immunohistochemistry was observed in the brush border membranes of the duodenum. This location was consistent with a hydrolase known as folate conjugase (folate hydrolase as a carboxypeptidase, not an endopeptidase)that had been described in the older literature, with the protein having been partially purified from the human small intestine. No cloning or sequencing of this gene had been done. There is a form of folate hydrolase that is found in all cells in the lysosomes and it was recently sequenced. There is no sequence relationship between the lysosomal endopeptidase. Membrane fraction of the LNCaP cells was very rich in folate hydrolase activity. The PSM specific monoclonal could be used to immunoprecipitate the folate hydrolase activity. This result always has the possibility that the folate hydrolase activity is not the same as PSM antigen but is a coprecipitating contaminant. Therefore PSM antigen was transfected into PC-3 cells. PC-3 cells do not express PSM nor do they have membrane folate hydrolase activity. In cells transfected with PSM antigen however expression of folate hydrolase activity was observed in the membranes. Thus PSM is a novel folate hydrolase,

folate carboxypeptidase, and is active in sequentially removing the terminal gamma-linked glutamates. In the proximal small intestine it is understandable why this enzyme would be in such a place, as the majority of folate available from food is polygammglutamated and this enzyme is responsible for its hydrolysis.

Materials: Methotrexate triglutamate (4-NH_2-10-CH_3-$PteGlu_4$ ($MTXglu_3$)), pteroylpentaglutamate ($PteGlu_5$), and para-aminobenzoylpentaglutamate, ($pABAGlu_5$) were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland) and samples were > 98% pure when evaluated by HPLC. N-acetyl-α-aspartylglutamate (NAAG) (40 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Protein A Sepharose 4 Fast Flow was purchased from Pharmacia (Piscataway, NJ). The 7E11-C5 monoclonal antibody to prostate specific membrane antigen was obtained from Cytogen Corporation, Princeton, NJ. All other reagents (p-hydroxymercuribenzoate, homocysteine, dithiothreitol (DTT), reduced glutathione) were of the highest purity commercially available from Sigma Chemical Co. (St. Louis, MO).

Culture and growth of human prostate adenocarcinoma cells (LNCaP, PC-3, TSU-Pr1, and Duke-145): LNCaP cells were maintained in defined culture medium, RPMI-1640 medium supplemented with non-essential amino acids, 5 mM glutamine, and 5% heat-inactivated fetal calf serum. Duke-145, PC-3, and TSU-Pr1 cells were grown in minimal essential medium (MEM), Ham's F-12K, and MEM, respectively, containing 5% fetal calf serum. No antibiotic was included in the media. Cells (1 x 10^6) were plated in T-75 tissue culture flasks containing 15 mL of medium and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Cell numbers were determined using a Model Z F Coulter Counter (Coulter

Electronic, Inc.). Prostate cells were harvested from plates by gentle scraping at 4 °C into phosphate buffered saline (136.9 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.34, PBS) and centrifuged at 500 X g to obtain a cell pellet. Sedimented cells were routinely rinsed twice with 15 mL volumes of PBS.

Transfection of PSM into PC-3 Prostate Cell Line: The full length 2.65 kb PSM cDNA was subcloned into a pREP7 eukaryotic expression vector (Invitrogen, San Diego, CA) as previously described. Plasmid DNA was purified from transfected DH5-α (Gibco-BRL) using a Qiagen maxi prep plasmid isolation kit (Qiagen Inc., Chatsworth, CA). Purified plasmid DNA (5 μg) was diluted with 300 μL of serum free RPMI media and mixed with 45 μL of lipofectamine (Gibco-BRL) which was previously diluted with 300 μL of serum free RPMI media to allow an DNA-liposome complex to form. The mixture was kept at room temperature for 30 minutes, then added to a 60 mm petri dish containing 60-70% confluent PC-3 cells in 2.4 mL serum free RPMI. The DNA-liposome complex containing serum free media was mixed gently to ensure uniform distribution and was then incubated for 6 h at 37 °C in a CO_2 incubator. Following incubation, the media containing liposome-DNA complex was aspirated and replaced with 6 mL of regular growth media (10% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamine). After 48 hours, cells were trypsinized and split 1:3 into 60 mm dishes containing regular media supplemented with 200 μg/mL of hygromycin B (Calbiochem, LaJolla, CA). Cells were maintained for 2 weeks with changes of media containing hygromycin B every third day until discrete colonies appeared. Colonies were isolated using a 6 mm cloning cylinder and were expanded in the same media. As a control, PC-3 cells were also transfected with the pREP7 vector alone.

Immunohistochemistry: The 7E11-C5 monoclonal antibody to prostate specific antigen was used. This antibody recognizes a portion of carbohydrate-containing peptide epitope on the amino terminal end of PSM that is located on the inner portion of the cytosolic membrane. After permeabilization of LNCaP and PC-3 transfected and non-transfected cells with a mixture of acetone and methanol (1:1 v/v) and blocking with 5% bovine serum albumin in 50 mM Tris buffered saline (TBS) pH 7.45, samples were incubated with 7E11-C5 antibody (20 μg/mL) for 1 h at room temperature. Negative controls were generated by substituting the same concentration of mouse IgG2ak for the PSM antibody. Using a secondary IgG_1 anti-mouse antibody conjugated with alkaline phosphatase, samples were re-incubated for 1 h, rinsed in TBS, and stained with bromochloroindolylphenol phosphate in 2-amino-2-methyl-1-propanol buffer. Cells expressing PSM demonstrate an intense blue staining.

Cell Membrane Preparation: Cell lysates were prepared by sonicating approximately 6 x 10^6 cells in 50 mM Tris pH 7.4 buffer (2 x 10 s pulses at 20 mWatts) in an ice-bath. Membrane fractions were obtained by centrifuging lysates at 100,000 x g for 30 mins. The supernatant fractions were saved and pelleted membranes were re-suspended by gentle trituration and re-sedimented at 100,000 x g for 30 mins through 10 mL of cold 50 mM Tris pH 7.4 buffer. Washed membrane fractions were dissolved in 50 mM Tris pH 7.4 buffer containing 0.1% Triton X-100 (Tris/Triton). Enzymatic activity and immunoprecipitation preparations were performed using this membrane preparation.

Immunoprecipitation of PSM from Membrane: Membrane pellets (~1 mg protein) solubilized in Tris/Triton buffer were incubated at 4 °C for 1 h in the presence of 7E11-C5 anti-prostate monoclonal antibody (6 ug

protein). Protein A Sepharose gel equilibrated in Tris/Triton buffer was added to the immunocomplex. This preparation was subsequently incubated for an additional hour at 4 °C. Sepharose beads were centrifuged at 500 x g for 5 mins and rinsed twice with Tris/Triton buffer at pH 7.4. Isolated beads were resuspended in 0.1 M glycine buffer pH 3.0, vortexed, and the supernatant fraction was assayed for hydrolase activity using $MTXglu_3$.

Pteroyl Gamma-Glutamyl Hydrolase Assay: Hydrolase activity was determined using capillary electrophoresis. The standard assay mixture contained 50 uM $MTXGlu_3$, 50 mM acetate buffer (pH 4.5) and enzyme to a final volume of 100 uL. A sample preparation without enzyme was incubated concurrently with enzymatic assays and reactions were conducted for times varying between 0 and 240 min at 37 °C. Activities were also determined in standard reaction mixture at varied pHs for 60 min. Reactions were terminated in a boiling water bath for 5 min and samples were stored frozen (-20 °C) until analysis. Following centrifugation (7,000 x g) to remove precipitated debris, capillary separation of MTX glutamated analogues was performed with a Spectra Phoresis 1000 instrument (Thermo Separation, San Jose, CA) with a 75 μm id x 50 cm silica capillary (Polymicro Technology, Phoenix, AZ). Separation of pteroyl(glutamate)$_n$ derivatives is achieved with an electrolyte of 20 mM sodium borate with 15 mM sodium dodecylsulfate (pH 9.5) with +20 Kev at 25 °C. Samples were applied hydrodynamically for 1-2 s and absorbance monitored at 300 nm. Data were recorded with an IBM computer using CE-1000 software (Thermo Separation).

Protein determination: Protein concentrations of isolated membrane or supernatant fractions were

determined by incubating diluted aliquots with BCA reagent (Pierce Chemical Co., Rockford, IL) at 37 °C for 30 min. The spectrophotometric quantitation of protein was conducted by determining the absorbance at 562 nm against bovine serum albumin standard.

Statistical Analysis: Data were analyzed by using the *Statgraphics version 4.0 program* (Statistical graphics Corporation, Rockville, MD) and where summarized are expressed as mean ± S.D. Student's unpaired *t* test was used to determine significance of differences.

Results:

Membrane fractions isolated from human prostate adenocarcinoma cells (LNCaP) were incubated using primarily $MTXglu_3$ as substrate. The time course of hydrolysis of the gamma-linked triglutamate derivative and the subsequent appearance of $MTXglu_2$, $MTXglu_1$, and MTX after 30, 60, 120, and 240 min of incubation are illustrated in Figure 82. The semipurified PSM antigen exhibits pteroyl poly gamma-glutamyl exopeptidase activity that progressively liberates all of the possible glutamates from $MTXGlu_3$ with accumulation of MTX.

The PSM antigen was immunoprecipitated in the presence of 7E11-C5 anti-prostate monoclonal antibody and the PSM antigen-antibody complex was adsorbed onto a Protein A Sepharose Gel column. Following twice washing of the sepharose beads with 2 mL volumes of buffer and re-solubilization of the antigen-antibody complex by adjusting the elution pH to 3.0, the supernatant fraction was assayed for hydrolase activity. Figure 55 shows the capillary electrophoretic separation of successively cleaved glutamyl moieties from $MTXglu_3$ after 0, 30, 60 and 240

min incubations. Results similar to these in Figure 82 were obtained using $pteglu_5$ with formation of folate ($pteglu_1$).

The optimum pH activity profiles of the immunoprecipitated PSM hydrolase from LNCaP cells and of the membrane fractions from PC-3 PSM-transfected and non-transfected (vector alone) cells are shown in Figure 57. The reaction was monitored as a function of pH from 2 to 10 after an 1 h incubation with $MTXglu_3$. The extent of reaction was expressed as the concentration of $MTXglu_2$ formed per mg protein. Although all reaction products were detectable as illustrated in Figure 56, $MTXglu_2$ was the predominant hydrolyzed species at incubation times ranging from 10 to 60 min. The pH profile of membrane fractions isolated from both LNCaP and PC-3 PSM-transfected cells are identical and exhibit two maxima of PSM hydrolase activity at pH 5 and 8 with no measurable activity above pH 10.

To determine whether non-PSM expressing human adenocarcinoma cell lines (PC-3, TSU-Pr1, and Duke-145) exhibit folate hydrolase activity, isolated membrane preparations from these cell lines were analyzed (Figure 83). The less differentiated, hormone refractory prostate cell lines (PC-3, TSU-Pr1, and Duke-145) exhibit no appreciable activity after 2 h incubations. These results are in agreement with previous findings that demonstrate neither a presence of a mRNA for PSM nor antigen immunoreactivity with 7E11-C5 in these cells.

In further studies in which the cDNA for PSM was transfected into non-PSM antigen expressing PC-3 cells, a close correlation between PSM antigen immunoreactivity and hydrolase activity was observed

with $MTXglu_3$ in membranes of LNCaP and PC-3 PSM-transfected cells (Figures 58 and 59). Immmunohistochemical analyses of LNCaP (Figure 58) and PSM antigen expressing PC-3 (Figure 85B) cells revealed distinct positive staining with 7E11-C5 anti-prostate monoclonal antibody. Figure 85C illustrates no immunoreactivity in PC-3 cells expressing the pREP7 hygromycin vector alone. In preparations of negative controls, all three cell lines were reacted with IgG2aK rather than with 7E11-C5 antibody. No background staining resulted with the secondary antibody conjugated with alkaline phosphatase.

To compare PSM hydrolase activity with that of other gamma-glutamyl hydrolases that either reside within the lysosome or are secreted as observed in several neoplastic cells, its reactivity in the presence of thiol-containing reducing agents, namely, reduced glutathione, homocysteine, and dithiothreitol (DTT), and the thiol reagent, p-hydroxymercuribenzoate (PHMB), at concentrations ranging from 0.05 - 0.5 mM was observed. Of the reduced sulfhydryl derivatives, it was discovered that only DTT ($\geq$ 0.2 mM) was slightly inhibitory (86 $\pm$ 3% of control). Unlike gamma-linked peptide hydrolase retained within the lysosome, PSM hydrolase activity was maintained in the presence of 0.5 mM PHMB.

The reactivity of PSM hydrolase against an α-glutamate dipeptide, N-acetyl-α-aspartylglutamate (NAAG), has been investigated and that the PSM enzyme from either LNCaP or PSM transfected PC-3 cell membranes hydrolyses NAAG producing N-acetylaspartate and glutamate was observed. Furthermore, $MTXglu_3$, $pteglu_5$, and $pABAglu_5$ were potent inhibitors of the PSM-mediated NAAG hydrolysis.

Discussion:

Membrane-bound PSM antigen has pteroyl poly gamma-glutamyl carboxypeptidase (folate hydrolase) activity. Gamma-glutamyl hydrolase activity is also present in lysosomes of cells and these enzymes may be responsible for regulating the length of exogenous and endogenous folyl polyglutamate chain lengths. A characteristic difference between these two hydrolases is that the PSM enzyme exhibits substantial activity at pH values 7.5 to 8.0 in addition to having an acidic pH 4.5 to 5 optimum. Moderate levels of hydrolase activity are present within LNCaP cytosolic compartment and may represent the short intracellular fragment of this class II enzyme. This reflects an interesting situation in these cells where the majority of RNA codes for the membrane-bound enzyme that is localized extracellularly. The ratio of the mRNAs in these samples that code for the class II membrane and the cytosolic proteins is ten to one. In normal prostate tissue, the mRNA coding for the membrane protein is only one-tenth that of the cytosolic form.

It is clear from this study that the prostate specific membrane antigen functions as a folate hydrolase and is unique in that it has activity on both the gamma-linked as well as the alpha linked peptide bonds. This is interesting for a number of reasons. First in the normal prostate it was demonstrated that the majority of the mRNA encodes a protein, PSM', that is likely to be cytosolic and would imply that it may be that in the prostate that folates could exists in the lesser glutamated species. If so then it means that the folate in the prostate can readily leak out and that the prostate may be subjected to "microenviromental folate deficiencies" This may be related to the high worldwide incidence of "microscopic prostate cancer" as folate deficiencies are associated with carcinogenesis

in a number of tissues.

Benign enlargement of the prostate and prostate cancer occur in older men. It also occurs that the uptake of folate decreases with aging. If folate uptake decreases with aging this may be due to decreased PSM folate hydrolase activity in the proximal intestine. To correct such a deficiency it might be possible to use PSM folate hydrolase in foods to release the folate before consumption or take it with foods as is done with lactase in lactose intolerant individuals. If the prostate in men is susceptible to folate depletion then nutritional supplementation may help reduce the development of the microscopic lesion, indeed in some cancers such as cancer of the colon, folate supplementation was found to reduce cancer formation.

Why would the prostate cells prefer to have the lesser glutamated forms of folate? It may be that methionine synthase which is an enzyme key to folate uptake and folate utilization for one carbon methyl transfer metabolism may utilize the nonglutamated folate preferentially. In addition to folate deficiency, choline and methionine deficiency is also associated with tumor development. If shown to modulate one carbon transfers, it might be useful to inhibit this enzyme as a means to inhibit cancer development and thus serve as a chemopreventative agent. Again modulation of PSM folate hydrolase may play a role in tumor prevention and modulation of tumor growth.

A feature that cell biologists use in transfecting DNA into cells often requires selection of the transfected gene and often multiple transfections are performed. These are done with drugs that are toxic to cells such as Hygromycin and use genes that code for Hygromycin resistance which are bacterial. It may be that PSM

could be used as a selectable marker by growing the transfected cells in folate free media and including polyglutamated folate which would be able to rescue cells from folate deficiency if they expressed PSM.

PSM folate hydrolase activity can possibly be used as a prodrug converting enzyme. In the normal prostate PSM is intracellular. In the transformed cell the majority of the protein and its attendant enzymatic activity is extracellular in location. It may be that as the enzymes associated with cell growth require the polyglutamated forms the cancer finds a way to remove PSM folate hydrolase from the interior by alternative splicing to an extracellular enzyme. PSM is a membrane protein and is found to predominate in cancer, but PSM' is likely a cytosolic protein which predominates in the normal condition.

This implies that development of a prodrug that requires metabolism before it can be taken up by the tumor cell could be activated by the PSM folate hydrolase which is predominate in the cancer.

Methotrexate triglutamate was one of the agents used to identify the enzymatic activity of PSM antigen. Methotrexate triglutamate would not be able to use the transport protein to be taken into tumor cells, because there are specific structural requirements for folate, or methotrexate transport. If one removes the gamma-linked glutamates then methotrexate can be taken into cells and can exerts its antifolate, antitumor growth action.

Therfore methotrexategammatriglutamate was used to examine the action of this compound on the in vitro growth of PC-3 cells transfected with a plasmid with a

selectable marker versus a plasmid with a selectable marker that expresses PSM antigen as well. the PC-3 cells that were transfected with PSM were inhibited 85% in growth by day four by 10uM methotrexate triglutamate, while the PC-3 plasmid only transfectants did not exhibit any significant inhibition of growth.

PSM's folate hydrolase activity hydrolyses down to the last glutamate which is in alpha linked position but does not remove it. Because it does not remove the last glutamate, PSM antigen's folate hydrolase activity better serves the prodrug activation requirements of such a prodrug. Also because it is a human enzyme it is less likely than the carboxypeptidase G2 will cause an immune response because PSM antigen is normally present in the body.

In addition PSM could also be used as part of a prodrug strategy that utilized gene transfer and a tissue or tumor specific promoter, say such that it would be linked to CEA promoter and PSM expressed in colon tumors and the patients subsequently given the prodrug such as methotrexate triglutamate. The same is also true for the protein itself, either the whole protein or the components of the active site or a modified version that would have increased prodrug activating activity could be linked to a delivery vehicle such as an antibody or other specific targeting ligand, delivered to the tumor for localization and subsequent activation.

Methotrexate as a prodrug may be enhanced in specificity by using alpha linked glutamates rather than gamma linked glutamates because the ubiquitous lysosomal hydrolase enzyme is specific for the gamma linked bond. A pro-drug with all alpha linked glutamates would not be a substrate, but would be a

substrate for the PSM folate hydrolase.

In addition to methotrexate a number of potential enzyme substrates can be employed as cytotoxic prodrugs. The synthesis of potential prodrugs, PALAglu, and a number of other potential agents are described.

Alpha-linked methotrexate material is synthesized by the following Merifield solid phase scheme (see Figure 88). The scheme is based on a modification of the standard Merifield solid peptide synthesis that was applied to the synthesis of methotrexate γ polyglutamates. In brief the N-Fmoc-4-terbutylglutamate is first connected to the resin under standard coupling conditions using diisoprpylazodicarboxylate as a coupling reagent. The Fmoc protecting group is then removed with piperidine, and this cycle would be reiterated for as many times as glutamates would be needed to obtain the desired analog. For example say the pentaglutamate on solid support is the intermediate required for the preparation of methotrexate-alpha-tetraglutamate. It is deprotected at the terminal nitrogen by treatment with piperidine, then coupled with pteroic acid analogue under the same conditions used above. The terbutyl and the resin are all removed in one step with 95% trifluoroacetic acid (TFA) to provide the desired material. This process is applied to every analog. The gamma linked material is provided in a similar manner for use comparative studies with the alpha-linked material (see figure 89). Because of the carboxypeptidase activity a number of combination of alpha and gamma linked acidic amino acid can be optimized for their utilization of the enzyme and for in vivo activity. In addition to the folate like antagonists, a number of amino acid analogs were found in the past to have antitumor activity but lacked in

vivo specificity. These agents are targetable by attaching a glutamate to the carboxy terminus of the amino acid as described and shown in the figures.

PALA-Glutamate 3 and analog 5, was synthesized in a similar manner with the addition to the introduction of a protected phosphonoacetate moiety instead of a simple acetate. It is compatible with the function of diethylphosphonoacetic acid which allows the removal of the ethyl groups under relatively mild conditions.

Commercially available diethylphosphonoacetic acid was treated with perfluorophenyl acetate in pyridine at 0 deg.C to room temperature for an hour to afford the corresponding pentafluorophenyl ester in nearly quantitative yield after short path column chromatography. This was then reacted with gamma-benzylaspartate and HOAT in tetrahydrofuran for half an hour at reflux temperature to give protected PALA 7 (N-phosphonoacetylaspartate) in 90% yield after flash column chromatography. The free acid was then activated as its pentafluorophenyl ester 8, then it was reacted with delta-benzyl-L-glutamate and HOAT in a mixture of THF-DMF (9:1, v/v) for 12 hours at reflux to give fully protected PALA-Glutamate 9 in 66% yield after column chromatography. Sequential removal of the ethyl groups followed by the debenzylation was accomplished for a one step deprotection of both the benzyl and ethyl groups. Hence protected PALA-Glutamate was heated up to reflux in neat trimethylsilylchloride for an overnight period. The resulting bistrimethylsilylphosphonate ester 10 was submitted without purification to hydrogenolysis (H_2, 30 psi, 10% Pd/C, ethylacetate). The desired material 3 was isolated after purification by reverse phase column chromatography and ion exchange resin.

Analogs 4 and 5 were synthesized by preparation of phosphonoglutamate 14 from the alpha-carboxyl-protected glutamate.

Commercially available alpha-benzyl-N-Boc-L-glutamate 11 was treated at refluxing THF with neat boranedimethylsulfide complex to afford the corresponding alcohol in 90% yield. This was transformed into bromide 12 by the usual procedure (Pph_3, CBr_4).

The Michaelis-Arbuzov reaction using triethylphosphite to give the corresponding diethylphosphonate 13 which would be deprotected at the nitrogen with trifluoroacetic acid to give free amine 14. The latter would be condensed separately with either pentafluorophenylesters 6 or 8 to give 16 and 15 respectively, under conditions similar to those described for 3. 15 and 16 would be deprotected in the same manner as for 3 to yield desired analogs 4 and 5.

An inhibitor of the metabolism of purines and pyrimidine like DON (6-diazo-5-oxo-norleucine) or its aspartate-like 17, and glutamate-like 18 analogs would be added to the series of substrates.

Analog 20 is transformed into compound 17 by treatment with oxalyl chloride followed by diazomethane and deprotection under known conditions to afford the desired analogs. In addition, azotomycin is active only after in vivo conversion to DON which will be released after action of PSM on analogs 17, 18, and 19.

Representative compounds, 21 and 22, were designed based on some of the specific effects and properties of PSM, and the unique features of some newly discovered cytotoxic molecules with now known mode of action. The

latter, referred to commonly as enediynes, like dynemycin A 23 and or its active analogs. The recent isolation of new natural products like Dynemycin A 23, has generated a tremendous and rapidly growing interest in the medical and chemical sciences. They have displayed cytotoxicities to many cancer cell lines at the sub-nanomolar level. One problem is they are very toxic, unstable, and non-selective. Although they have been demonstrated, in vitro, to exert their activity through DNA damage by a radical mechanism as described below, their high level of toxicity might imply that they should be able to equally damage anything in their path, from proteins to enzymes.

These molecules possess unusual structural features that provide them with exceptional reactivities. Dynemycin A 23 is relatively stable until the anthraquinone moiety is bioreduced into hydroanthraquinone 24. This triggers a chain of events by which a diradical species 25 is generated as a result of a Bergman cycloaromatization[f]. Diradical species 25 is the ultimate damaging edge of dynemycin A. It subtracts 2(two) protons from any neighboring molecule or molecules(ie. DNA) producing radicals therein. These radicals in turn combine with molecular oxygen to give hydroperoxide intermediates that, in the case of DNA, lead to single and double strand incision, and consequent cell death. Another interesting feature was provided by the extensive work of many organic chemists who not only achieved the total synthesis of (+)-dynemycin A 23 and other enediynes. but also designed and efficiently prepared simpler yet as active analogs like 26.

Enediyne 26 is also triggerable and acts by virtue of the same mechanism as for 23. This aspect is very relevant to the present proposed study in that 27 (a

very close analog of 26) is connected to NAAG such that the NAAG-27 molecule, 21, would be inert anywhere in the body (blood, organs, normal prostate cells) except in the vicinity of prostate cancer, and metastatic cells. In this connection NAAG plays a multiple role:

- Solubilization and transport: analogs of 26-type are hydrophobic and insoluble in aqueous media, but with a water soluble dipeptide that is indigenous to the body, substrate 21 should follow the ways by which NAAG is transported and stored in the body.

- Recognition, guidance, and selectivity: Homologs of PSM are located in the small intestines and in the brain.

In the latter, a compound like 27 when attached to a multiply charged dipeptide like NAAG, has no chance of crossing the blood brain barrier. In the former case, PSM homolog concentration in the small intestines is in the brush border and not likely to be exposed to prodrugs in the serum. In addition, one could enhance the selectivity of delivery of the prodrug by local injection in the prostate.

26 and its analogs are established active molecules that portray the activity of dynemycin A. Their syntheses are described in the literature. The total synthesis of optically active 27 has been described[6]. The synthetic scheme that for the preparation of 28 is almost the same as that of 27. However, they differ only at the position of the methoxy group which is meta to the nitrogen in the case of 28. This requires an intermediate of type 29 prepared by modification of the Myers' method.

Since NAAG is optically pure, its combination with

racemic material sometimes complicates purification of intermediates. In addition, to be able to modify the components of this system one at a time, optically pure intermediates of the type 21 and 22 are prepared. 27 was prepared in 17 steps starting from commercially available material. Another interesting feature of 27 is demonstrated in a very close analog 26, it possesses two(2) triggers as shown by the arrows.

The oxygen and the nitrogen can both engender the Bergman cycloaromatization and hence the desired damage. The simple protection deprotection manipulation of either functionality should permit the selective positioning of NAAG at the nitrogen or at the oxygen centers. PSM should recognize the NAAG portion of 21 or 22, then it would remove the glutamic acid moiety. This leaves 27 attached to N-acetylaspartate.

Intramolecular assisted hydrolysis of systems like N-acetylaspartyle is well documented in the literature. The aminoacid portion should facilitate the hydrolysis of such a linkage. In the event this would not work when NAAG is placed on the nitrogen, an alternative would be to attach NAAG to the oxygen giving rise to phenolic ester 22 which is per se labile and removable under milder conditions. PSM specific substrates can be designed that could activate pro-drugs at the site of prostatic tumor cells to kill those cells.

EXAMPLE 13:

GENOMIC ORGANIZATION OF PSM EXON/INTRON JUNCTION SEQUENCES

RNA is synthesized and then processed by having variable numbers of variable sized fragments cut out and remain in the nucleus (introns) and the remaining fragments (exons) joined together and transported out of the nucleus (mRNA) for use in translation into protein in the cytoplasm. This mRNA is what make the unique protein products of the cell, proteins of specialized cells are often made in a great abundance as are their respective coding mRNA's. These tissue specific mRNA's can be reverse transcribed (RT)into DNA by reverse transcriptase and amplified for detection by polymerase chain reaction (PCR) technology and thus the technique is called RT-PCR. If DNA is a contaminant of the MRNA fraction it would contain the message even though it was not being transcribed.

Knowledge of the intron exon junctions allows for the selection of primer pairs that cross an intron junction and thus allow the determination of DNA contamination of the RNA preparation, if present. If the intron junction were large it would be unlikely to be amplified with primers, while if the intron junction were small it would still produce a fragment that would be much larger than the predicted fragment size which is based on the cDNA sequence. Thus knowledge of the intron/exon junctions provides a control to determine if the RT-PCR product is contaminated with DNA. Another form of DNA that could also be amplified undesirably if present as a contaminant are pseudo genes, which are intronless forms of the mRNA that reside as DNA but are not expressed as RNA. Thus,

optimized primers for detection of PSM mRNA in samples would preferably contain sequences hybridizing across the intro/exon junctions which are as follows:

EXON 1 Intron 1

1F. strand
CGGCTTCCTCTTCGG
cggcttcctcttcgg taggggggcgcctcgcggag...tatttttca

1R. strand ...ataaaaagtCACCAAA

Exon 2 Intron 2

2F. strand
ACATCAAGAAGTTCT
acatcaagaagttct caagtaagtccatactcgaag...

2R. strand ...caagtggtcATATATTAAAATG

Exon 3 Intron 3

3F. strand
GAAGATGGAAATGAG
gaagatggaaatgag gtaaaatataaataaataaataa...

3R. ...TAAAAGTTGTGTAGT

Exon 4 Intron 4

4F. strand
AAGGAATGCCAGAGG
aaggaatgccagagg taaaaacacagtgcaacaaa...

4R. strand ...agagttgCCGCTAGATCACA

Exon 5 Intron 5

5F. strand

CAGAGGAAATAAGGT

cagaggaaataaggt aggtaaaaattatctttttt...

5R. strand ...gtgttttctATTTTTACGGGT

Exon 6 Intron 6

6F. strand

GTTACCCAGCAAATG

gttacccagcaatg gtgaatgatcaatccttgaat...

6R. strand ...aaaaaaagtTTATACGAATA

Exon 7 Intron 7

7F. strand

ACAGAAGCTCCTAGA

acagaagctcctaga gtaagtttgtaagaaaccargg...

7R. strand ...aaacacaggttatcTTTTTACCCA

Exon 8 Intron 8

8F. strand

AAACTTTTCTACACA

aaacttttctacaca gttaagagactatataaatttta...

8R. strand ....aaacgtaatcaTTTTCAGTTCTAC

Exon 9 Intron 9

9F. strand

AGCAGTGGAACCAG

agcagtggaaccag gtaaaggaatcgtttgctagca...

9R. strand ...aaagaTGTCTATACAGTAA

Exon 10 Intron 10

10F. Strand

CTGAAAAAGGAAGG

ctgaaaaaggaagg taatacaaacaaatagcaagaa...

Exon 11 Intron 11

11F. Strand

TGAGTGGGCAGAGG

agagg ttagttggtaatttgctataatata...

Exon 12 Intron 12

12F. strand

ATCTATAGAAGG

gtagtttcct gaaaaataagaaaagaatagat...

Exon 13 Intron 13

13F. strand

CTAACAAAAGAG

agggcttttcagct acacaaattaaaagaaaaaaag...

Exon 14 Intron 14

14F. strand

GTGGCATGCCCAGG

gtggcatgcccagg taaataaatgaatgaagtttcca...

Exon 15 Intron 15

15F. strand

CTAAAAATTGGC

aatttgtttgtttcc tacagaaaaaacaacaaaaca...

Exon 16 Intron 16

16F. strand

CAGTGTATCATTTG

cagtgtatcatttg gtatgttacccttccttttttcaaatt...

16R. strand ...aaagtcTAAGTGAAAA

Exon 17 Intron 17

17F. strand

TTTGACAAAAGCAA

tttgacaaaagcaa gtatgttctacatatatgtgcatat...

17R. strand ...aaagagtcGGGTTATCAT

Exon 18 Intron 18

18F. strand

GGCCTTTTTATAGG

ggccttttttatagg taaganaagaaaatatgactcct...

18R. strand ...aatagttgGTACAGTAGATA

Exon 19 Intron 19

19F. strand

GAATATTATATATA

gaatattatatata gttatgtgagtgtttatatatgtgtgt...

Notes: F: Forward strand

R: Reverse strand

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What is claimed is:

1. An isolated nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen.

2. An isolated DNA of claim 1.

3. An isolated cDNA of claim 2.

4. An isolated RNA of claim 1.

5. An isolated DNA of claim 2 operatively linked to a promoter of RNA transcription.

6. A vector which comprises the nucleic acid of claim 1.

7. A host vector system for the production of a polypeptide having the biological activity of the alternatively spliced prostate-specific membrane antigen which comprises the vector of claim 6 and a suitable host.

8. A host vector system of claim 7, wherein the suitable host is a bacterial cell, insect cell, or mammalian cell.

9. An isolated nucleic acid comprising a promoter sequence normally associated with the transcription of a gene encoding a human prostate-specific membrane antigen.

10. An isolated polypeptide having the biological activity of an alternatively spliced prostate-specific membrane antigen.

11. An antibody which specifically binds to the polypeptide of claim 10.

12. The antibody of claim 11, wherein the antibody is monoclonal antibody.

13. The antibody of claim 11, wherein the antibody is polyclonal antibody.

14. The antibody of claim 11, wherein the antibody is labelled with a detectable marker.

15. The labelled antibody of claim 14, wherein the marker is radioactive, or colorimetric, luminescent, or fluorescent marker.

16. A method of detecting in a sample the presence of a nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen which comprises: a) obtaining a suitable sample; b) extracting RNA from the sample; c) contacting the RNA with reverse transcriptase under suitable conditions to obtain a cDNA; d) contacting the cDNA under hybridizing conditions with two oligonucleotide primers,

i) the first primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 3' of nucleotide 114 of such DNA sequence, with the proviso that the 3' end of the primer does not hybridize to any sequence located 5' of nucleotide 114, and

ii) the second primer being capable of specifically hybridizing to a sequence

within a DNA sequence encoding prostate specific membrane antigen located immediately 5' of nucleotide 381 of such DNA sequence, with the proviso that the 5' end of the primer does not hybridize to any sequence located 3' of nucleotide 381;

d) amplifying any cDNA to which the primers hybridize to so as to obtain amplification product; e) determining the size of the amplification product; f) comparing the size of the amplification product to the size of the amplification product known to be obtained using the same primers with a non alternatively spliced human prostate specific membrane antigen, wherein a smaller amplification product is indicative of the presence of the alternatively spliced prostate specific membrane antigen, so as to thereby detect the presence of the alternatively spliced human prostate-specific membrane antigen in the sample.

17. A method of detecting a prostate tumor cell in a subject which comprises: which comprises: a) obtaining a suitable sample; b) extracting RNA from the sample; c) contacting the RNA with reverse transcriptase under suitable conditions to obtain a cDNA; d) contacting the cDNA under hybridizing conditions with two oligonucleotide primers,

i) the first primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 3' of nucleotide 114 of such DNA sequence, with the proviso that the 3'

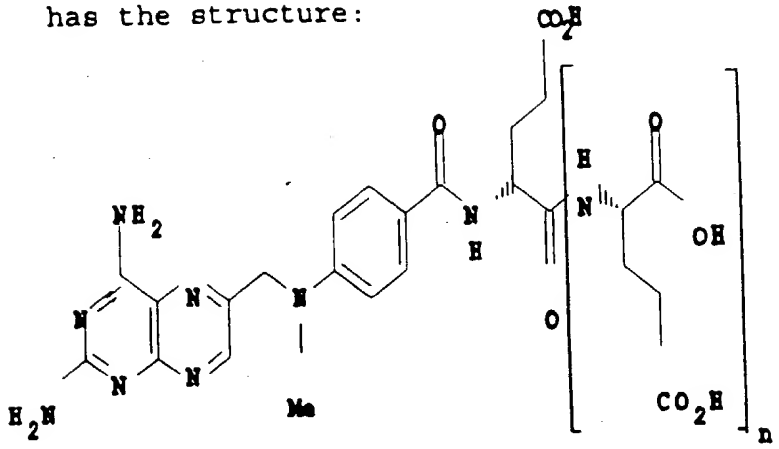
end of the primer does not hybridize to any sequence located 5' of nucleotide 114, and

ii) the second primer being capable of specifically hybridizing to a sequence within a DNA sequence encoding prostate specific membrane antigen located immediately 5' of nucleotide 381 of such DNA sequence, with the proviso that the 5' end of the primer does not hybridize to any sequence located 3' of nucleotide 381;

d) amplifying any cDNA to which the primers hybridize to so as to obtain amplification product; e) determining the amount of the amplification product; f) comparing the amount of the amplification product to the amount of the amplification product known to be obtained using the same primers with a non alternatively spliced human prostate specific membrane antigen, wherein a greater amount of the prostate specific membrane antigen is indicative of a prostate tumor cell in the subject, so as to thereby detect prostate tumor cell in the subject

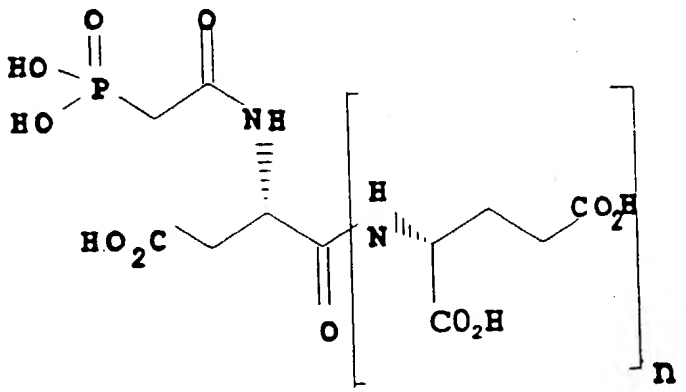
18. A compound comprising a conjugate of a cytotoxic agent and one or more amino acid residues, wherein each amino acid residue is glutamate or aspartate.

19. The compound of claim 18, wherein the compound has the structure:



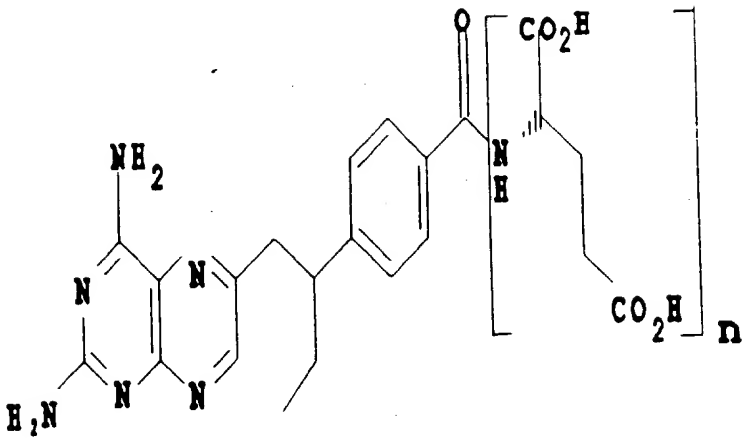
wherein n is an integer from 1-10 inclusive.

20. The compound of claim 18, wherein the compound has the structure:



wherein n is an integer from 1-10 inclusive.

21. The compound of claim 18, wherein the compound has the structure:



wherein n is an integer from 1-10 inclusive.

22. A pharmaceutical composition comprising the compound of any of claims 18-21 in a therapeutically effective amount and a pharmaceutically acceptable carrier.

23. A method of making prostate cells suseptible to a cytotoxic chemotherapeutic agent, which comprises contacting the prostate cells with an the compound of any claims 18-21 in an amount effective to render the prostate cells suseptible to the cytotoxic chemotherapeutic agent.

PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

ABSTRACT OF THE INVENTION

This invention provides an isolated nucleic acid molecule encoding an alternatively spliced human prostate-specific membrane antigen. This invention provides an isolated nucleic acid comprising a promoter sequence normally associated with the transcription of a gene encoding a human prostate-specific membrane antigen. This invention provides an isolated polypeptide having the biological activity of an alternatively spliced prostate-specific membrane antigen.

This invention provides a method of detecting a nucleic acid encoding an alternatively spliced human prostate-specific membrane antigen and a method of detecting a prostate tumor cell in a subject.

Lastly, this invention provides a pharmaceutical composition comprising a compound in a therapeutically effective amount and a pharmaceutically acceptable carrier and a method of making prostate cells susceptible to a cytotoxic chemotherapeutic agent.

FIGURE 1A

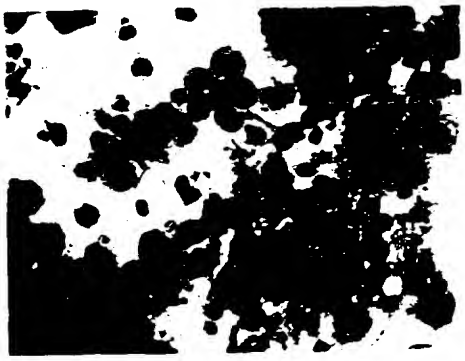


FIGURE 1B

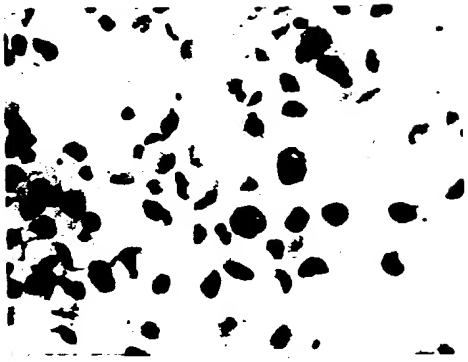
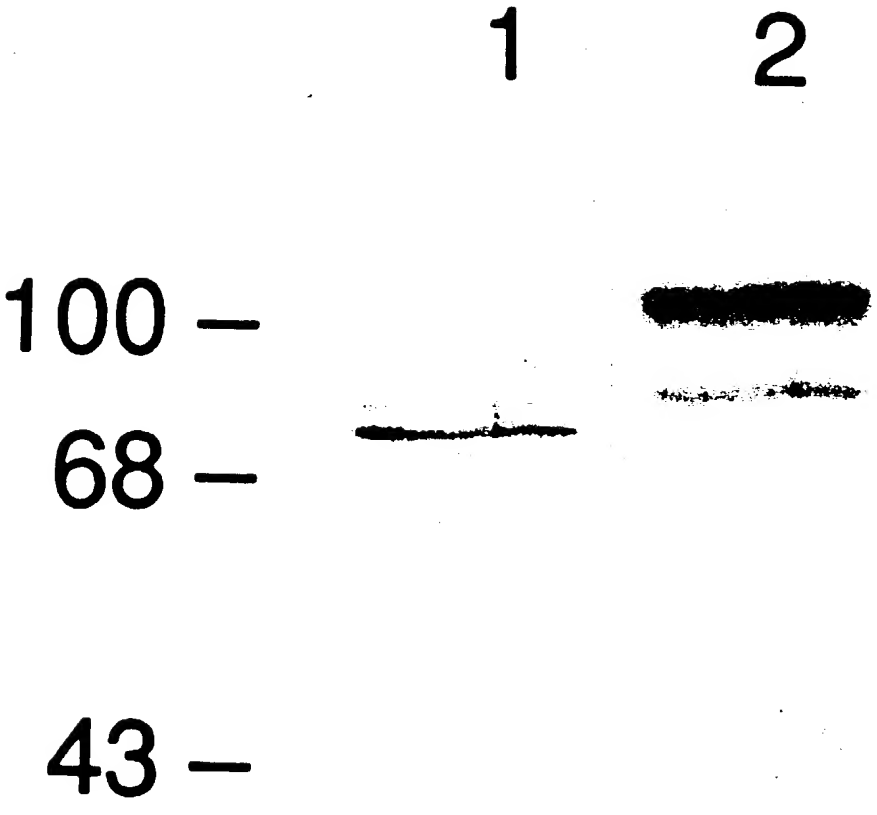


FIGURE 1C

FIGURE 2



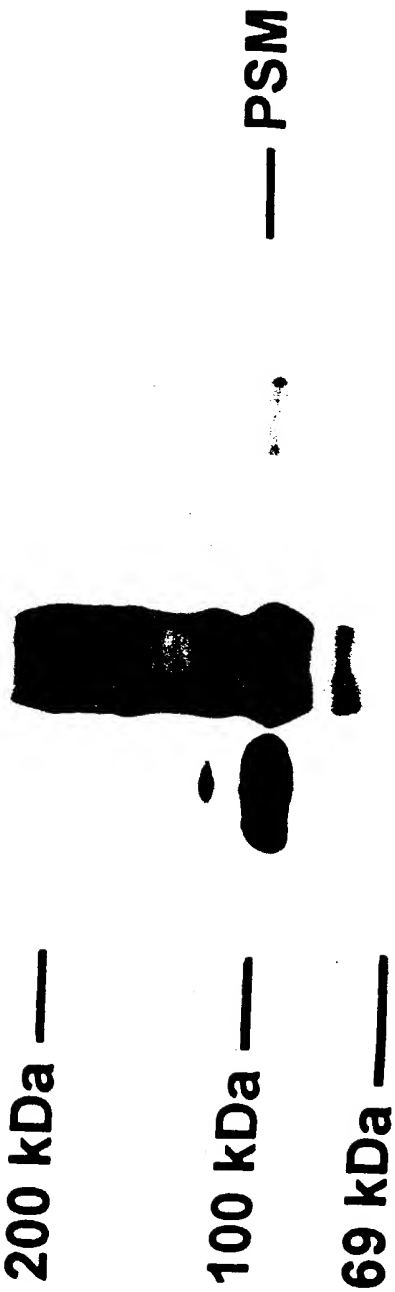


FIGURE 3

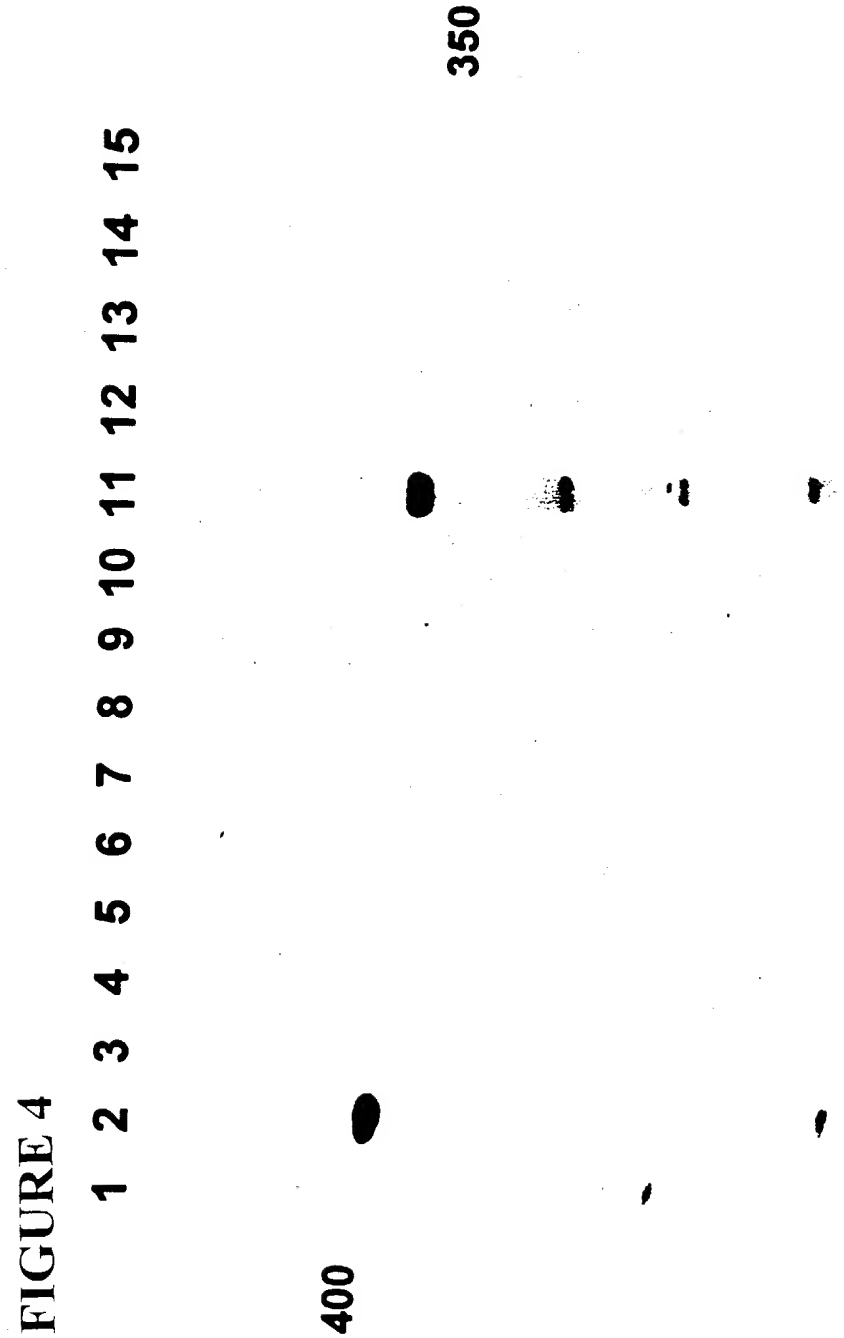


FIGURE 4

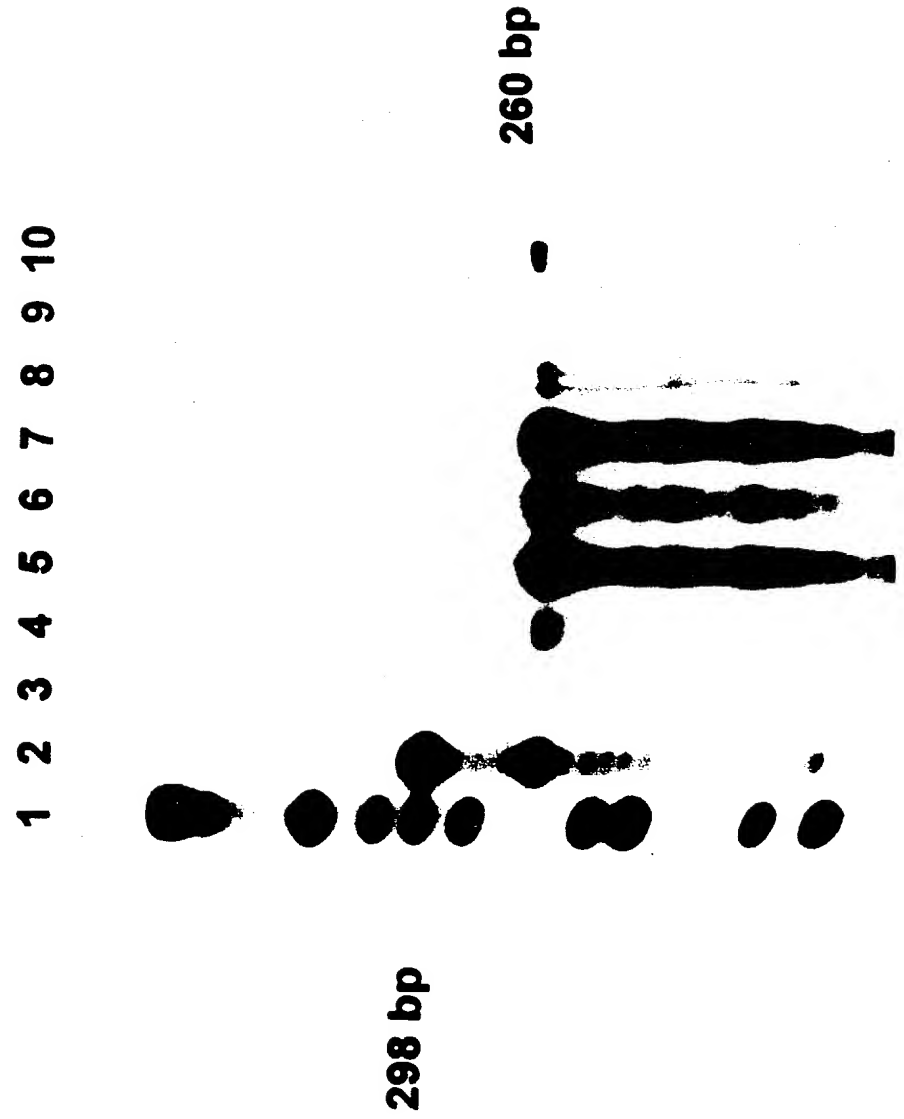


FIGURE 5

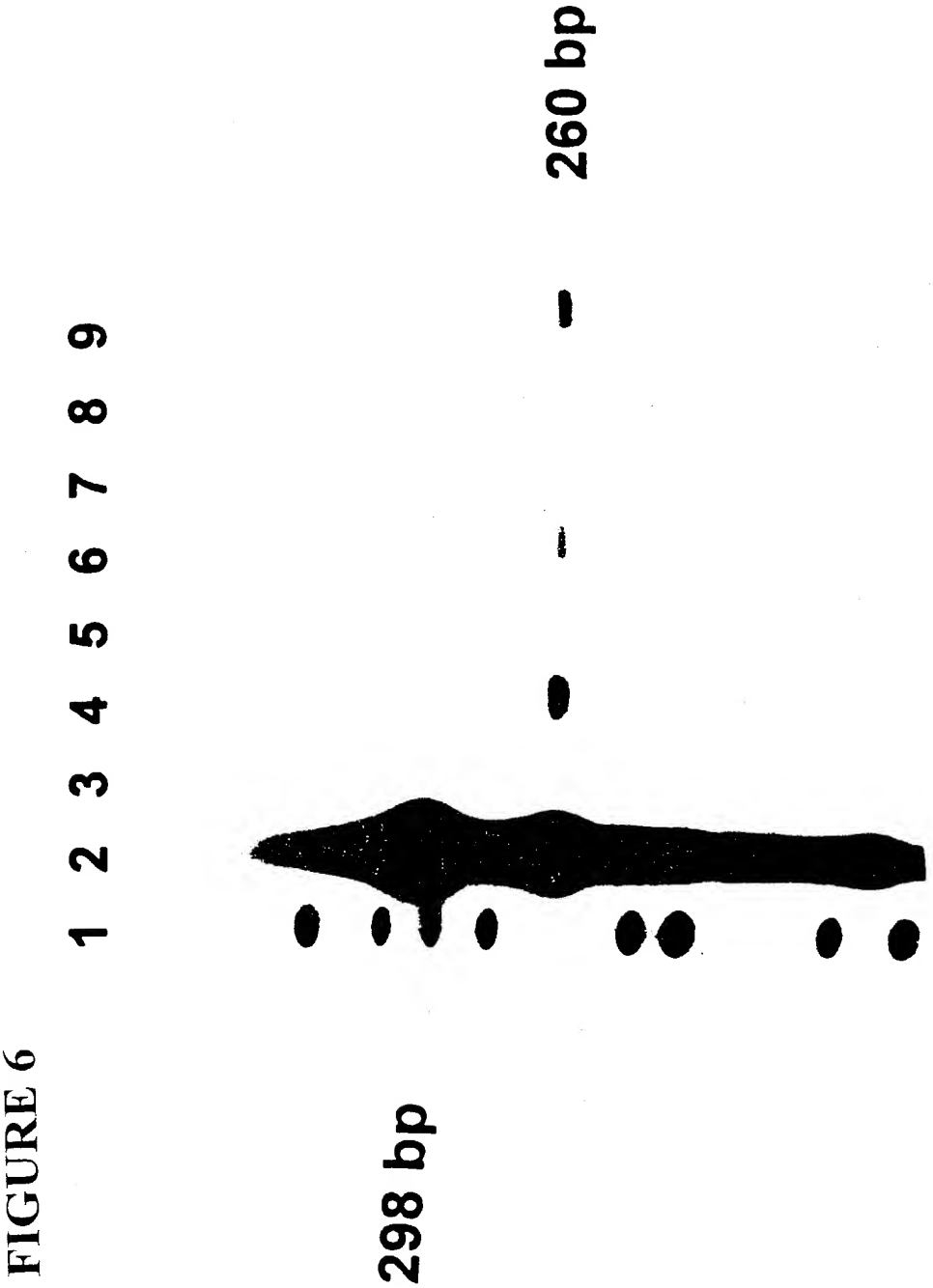


FIGURE 6

FIGURE 7

CELL LINE/TYPE	11p11.2-13 REGION	METASTATIC	PSM RNA DETECTED	PSM DNA DETECTED
LNCap			++	ND
HUMAN PROSTATE			++	ND
A9 (FIBROSARCOMA)	NO	NO	–	–
A9(11) (A9+HUM. 11)	YES	NO	–	REPEAT
AT6.1 (RAT PROSTATE)	NO	YES	–	–
AT6.1-11-c11	YES	NO	+	++
AT6.1-11-c12	NO	YES	–	–
R1564 (RAT MAMMARY)	NO	YES	–	–
R1564-11-c14	YES	YES	–	+
R1564-11-c15	YES	YES	–	REPEAT
R1564-11-c16	YES	YES	–	ND
R1564-11-c12	YES	YES	ND	+

FIGURE 8A

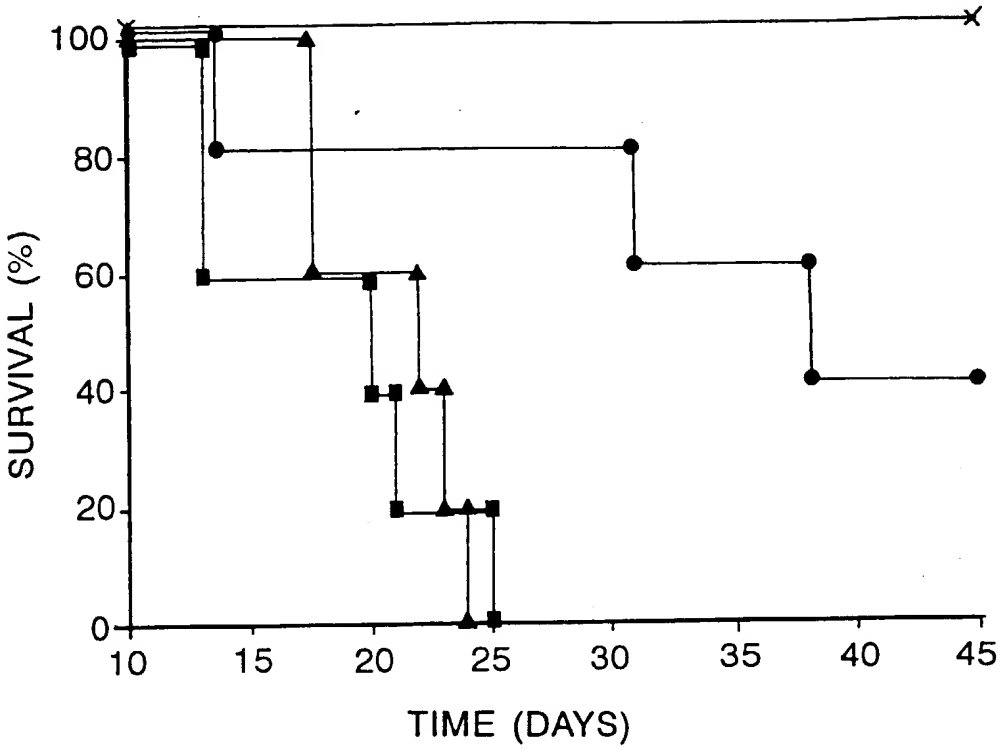


FIGURE 8B

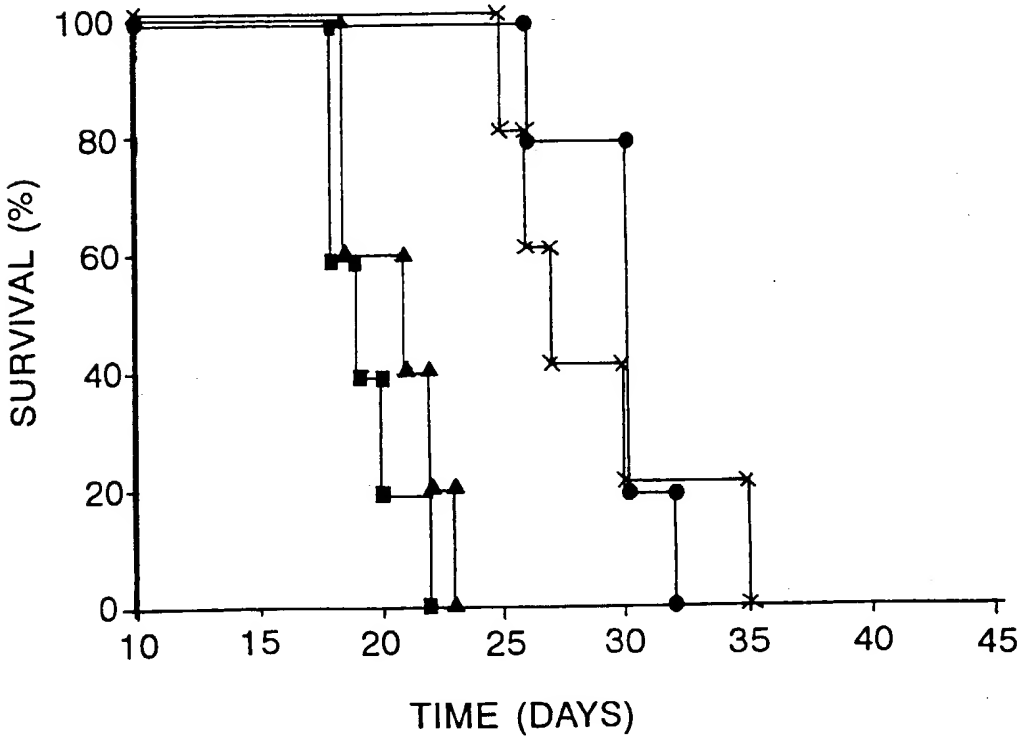


FIGURE 9A

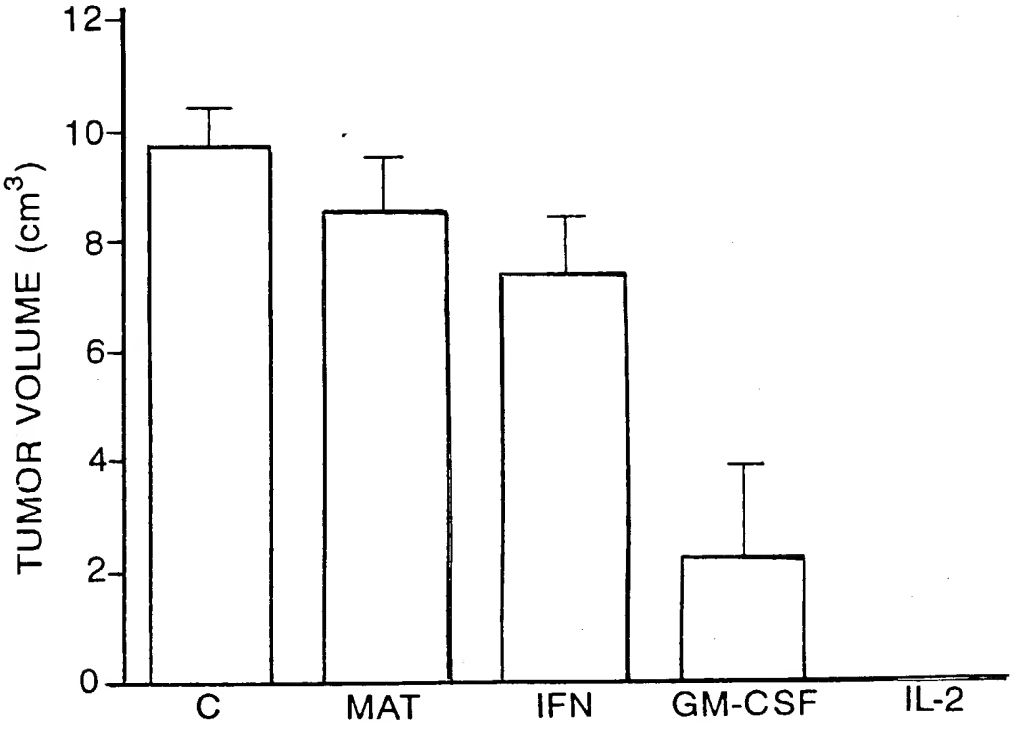
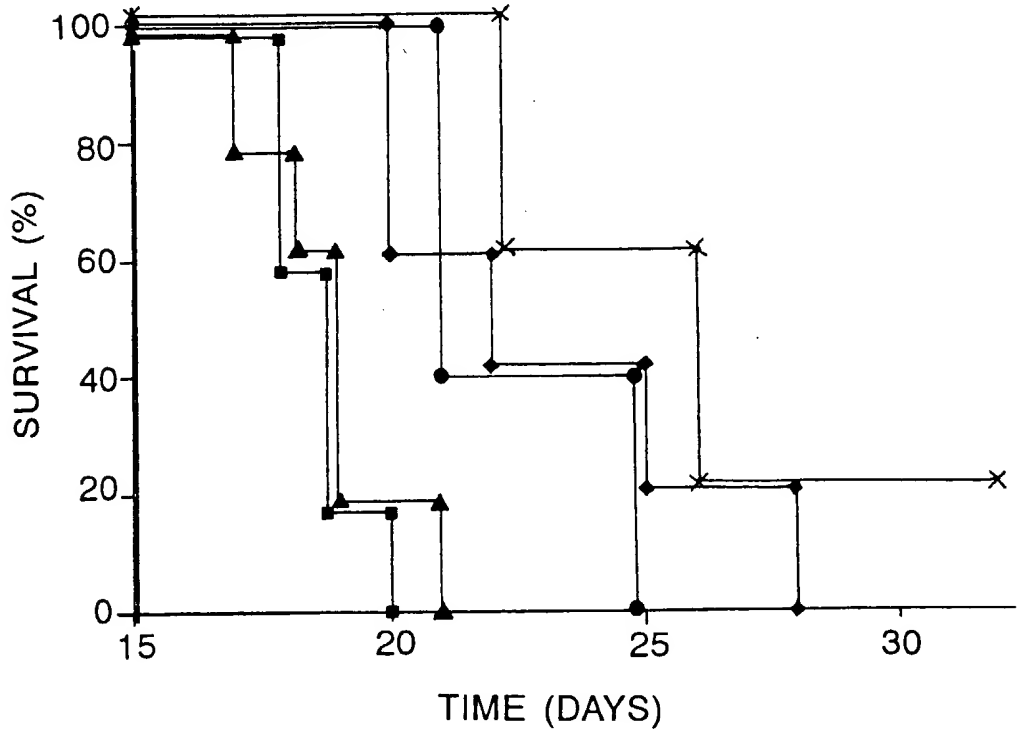


FIGURE 9B



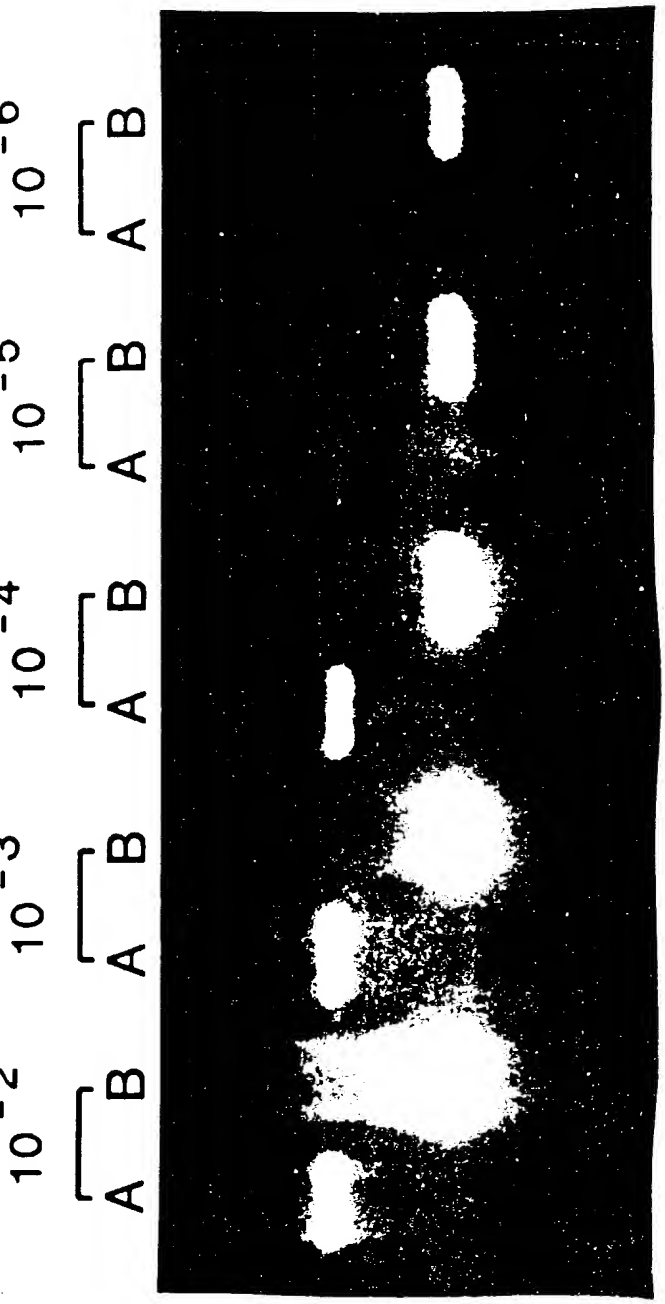


FIGURE 10

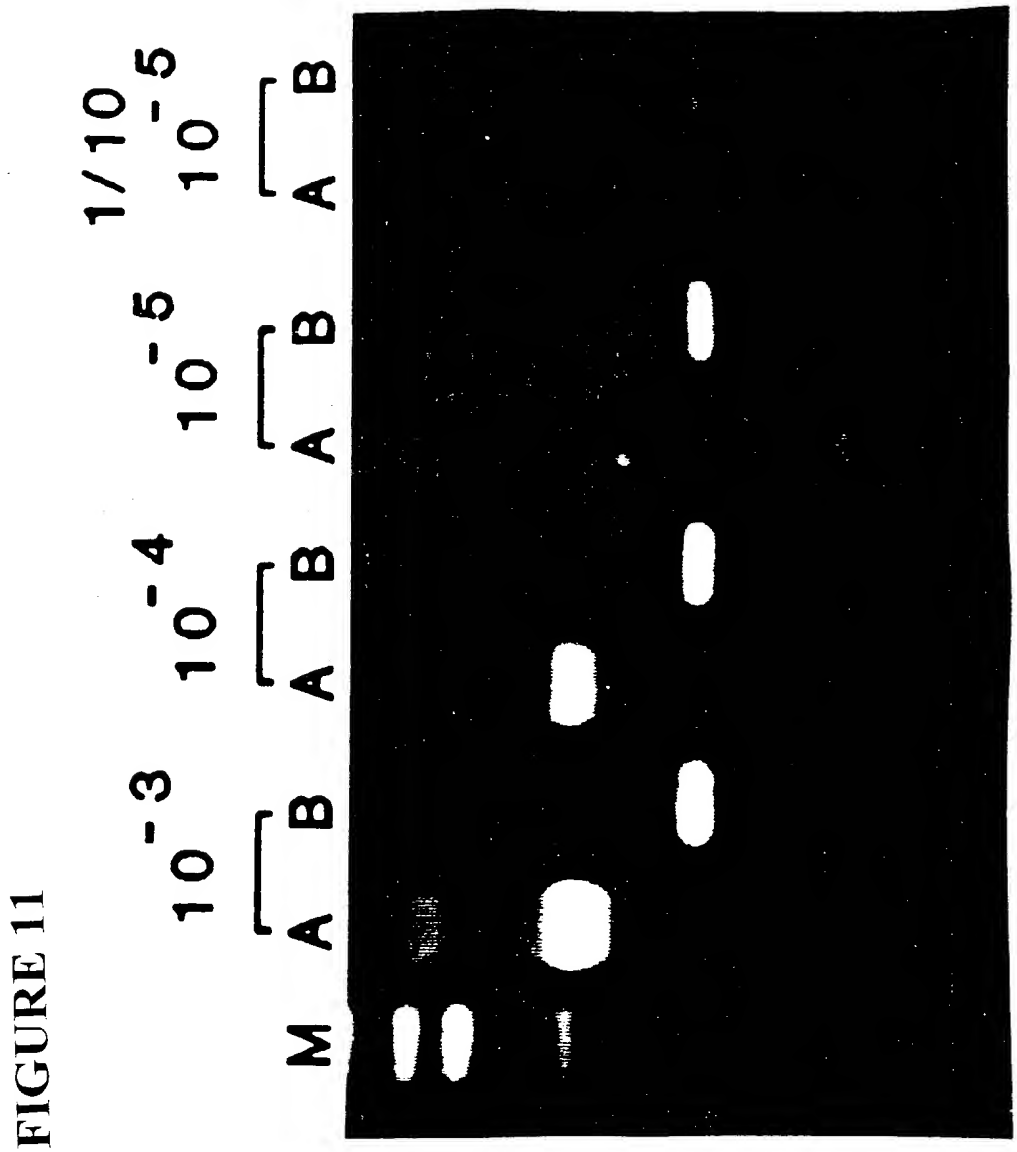


FIGURE 11

FIGURE 12

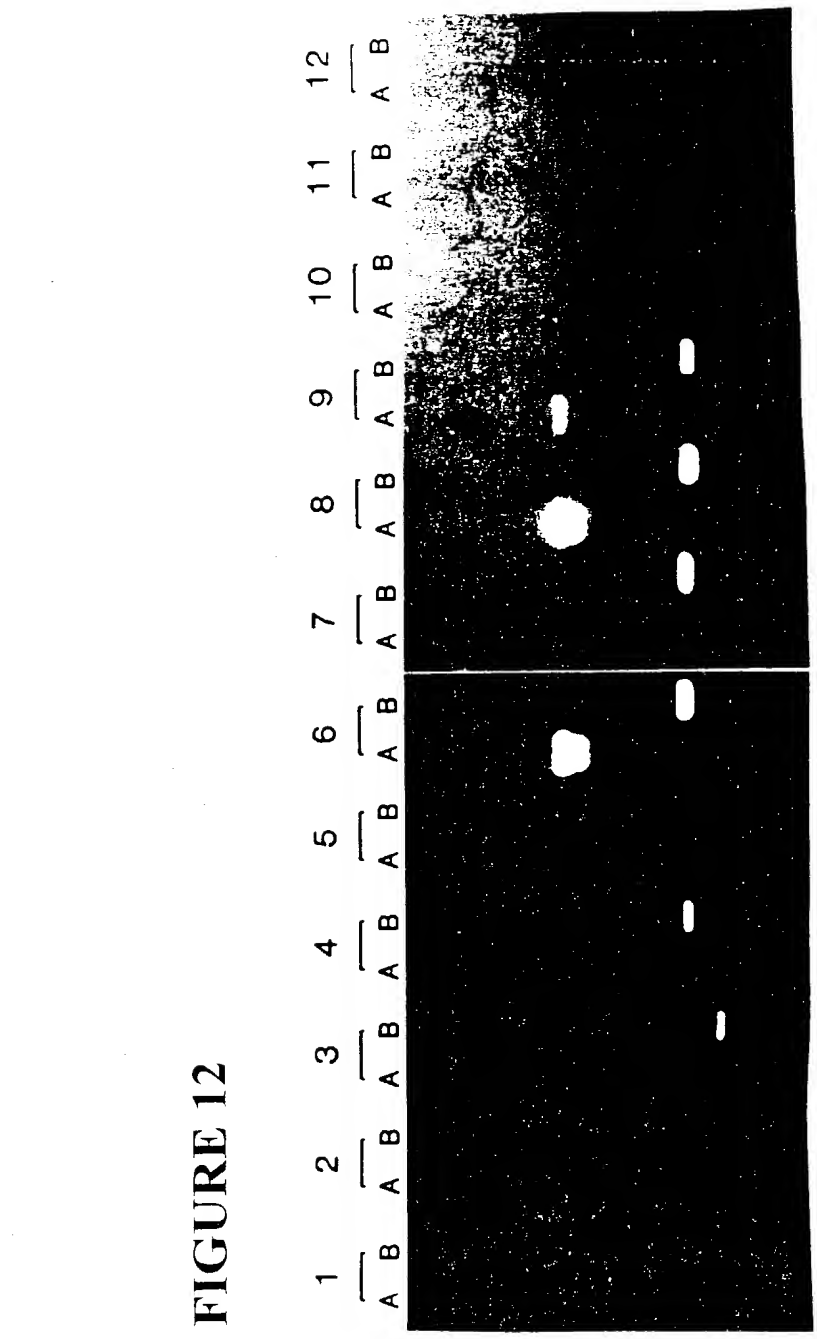


FIGURE 13

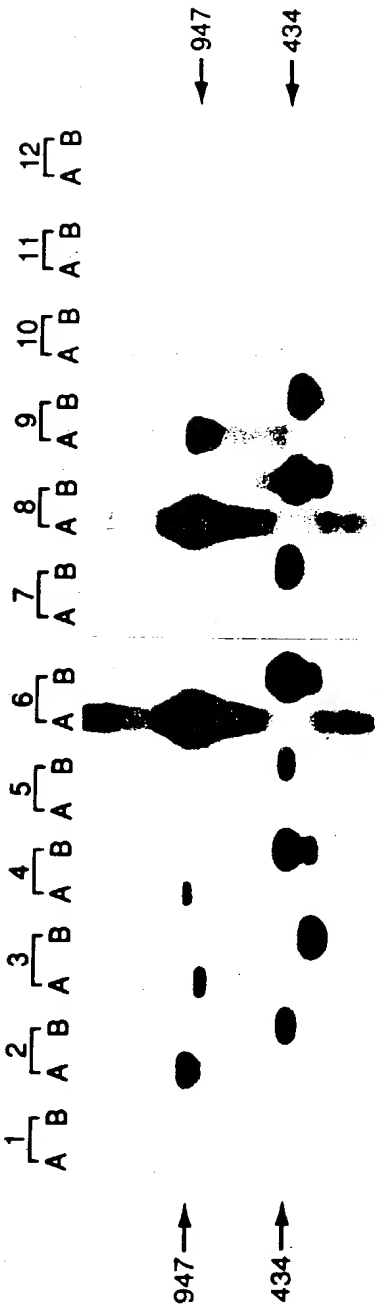

947
434
1 A B
2 A B
3 A B
4 A B
5 A B
6 A B
7 A B
8 A B
9 A B
10 A B
11 A B
12 A B
947
434

FIGURE 14

Patient	Stage	Treatment	PSA	PAP	PSA-PCR	PSM-PCR
1	T2NxMo	None	8.9	0.7	–	+
2	T2NoMo	RRP 7/93	6.1	–	–	+
3	T2CNoMo	PLND 5/93	4.5	0.1	–	+
4	T2BNoMo	RRP 3/92	NMA	0.4	–	+
5	T3NxMo	Proscar + Flutamide	51.3	1.0	–	+
6	Recur T3	I-125 1986	54.7	1.4	–	+
7	T3ANoMo	RRP 10/92	NMA	0.3	–	+
8	T3NxMo	XRT 1987	7.5	0.1	–	–
9	T3NxMo	Proscar + Flutamide	35.4	0.7	–	–
10	D2	S/P XRT Flutamide +Emcyt	311	4.5	+	+
11	D2	RRP 4/91 Lupron 10/92 Velban + Emcyt 12/92	1534	1.4	+	+
12	T2NoMo	RRP 8/91	NMA	0.5	–	+
13	T3NoMo	RRP 1/88 Lupron + Flutamide 5/92	0.1	0.3	–	–
14	D1	PLND 1989 XRT 1989	1.6	0.4	–	–
15	D1	Proscar + Flutamide	20.8	0.5	–	–
16	T2CNoMo	RRP 4/92	0.1	0.3	–	–

FIGURE 15A

```
          10         20         30         40         50         60
          |          |          |          |          |          |
  1 GCGCCTTAAA AAAAAAAAAC TTTCTTGGAA AATGTCCAGC TCTTGCTTAA ATATAAAAAT
    CGCGGAATTT TTTTTTTTTG AAAGAACCTT TTACAGGTCG AGAACGAATT TATATTTTTA

 61 GAAAGGAAGA AAGAGACTCT CCTCTCTCCA CTCCTATAAT TATGAGGAAC TTTTATTCAA
    CTTTCCTTCT TTCTCTGAGA GGAGAGAGGT GAGGATATTA ATACTCCTTG AAAATAAGTT

121 CTCTGAAATT CTATACAATC TCTACAATAC TCTACTGAAT AAAAGCAGAG CAGAAAAAGC
    GAGACTTTAA GATATGTTAG AGATGTTATG AGATGACTTA TTTTCGTCTC GTCTTTTTCG

181 TGCGCTTTTT TTCCATAGTC GGGAATGCTT GTCATCAGTG TAAATCACCA CCGCGCCCTT
    ACGCGAAAAA AAGGTATCAG CCCTTACGAA CAGTAGTCAC ATTTAGTGGT GGCGCGGGAA

241 TTTCCTAAAG AATATTATTG TTATTAATAA ACATGTAGGG TATTATCCTC CACTTACATT
    AAAGGATTTC TTATAATAAC AATAATTATT TGTACATCCC ATAATAGGAG GTGAATGTAA

301 ACAAAACCAT TTTTTAAAGC CGGGCGTGGT GGCTCACGCC TGTAATCCCA GCACTTTGGG
    TGTTTTGGTA AAAAATTTCG GCCCGCACCA CCGAGTGCGG ACATTAGGGT CGTGAAACCC

361 AGGCCCAGAC AGGCGGATCA CGAAGTCGAG AAATCGAGAC CATCCTGGCC AACATGGTGA
    TCCGGGTCTG TCCGCCTAGT GCTTCAGCTC TTTAGCTCTG GTAGGACCGG TTGTACCACT

421 AACCCCATCT CTACTAAAAA TACAAAAATT AGCTGGGCGT GGTGGCGGGC TCCTGTAGTC
    TTGGGGTAGA GATGATTTTT ATGTTTTTAA TCGACCCGCA CCACCGCCCG AGGACATCAG

481 CCAGCTACTC AGGAGGCTGA GGCAGGAGAA TCGCTTGAAC CGGGGAGGCG GAGGTTGCAG
    GGTCGATGAG TCCTCCGACT CCGTCCTCTT AGCGAACTTG GCCCCTCCGC CTCCAACGTC

541 TCAGCCAAGA TAGCGCCACT GCACTGGAGC CTGGTGACAG AGTGAGACTC CCTCAAGAAA
    AGTCGGTTCT ATCGCGGTGA CGTGACCTCG GACCACTGTC TCACTCTGAG GGAGTTCTTT

601 GAAAGGAAGG GAAGGGAAAG GGAAGGAAGG GGAGGGGAAG GGAGGGGAGG GGAGGGGAGG
    CTTTCCTTCC CTTCCCTTTC CCTTCCTTCC CCTCCCCTTC CCTCCCCTCC CCTCCCCTCC

661 AAAGAAAAGA ATACTGGAAC TTGTTGAAGG CAGAGACTTT ATTTTCATAT CCCGGCTATG
    TTTCTTTTCT TATGACCTTG AACAACTTCC GTCTCTGAAA TAAAAGTATA GGGCCGATAC

721 TCTGGCTACT GTCTTACGTA ATAGATATAA AATCAATCTT GGTTGGATTA ACCAGAAGAA
    AGACCGATGA CAGAATGCAT TATCTATATT TTAGTTAGAA CCAACCTAAT TGGTCTTCTT
```

FIGURE 15B

```
781 TGAGAAGATA TATTCTGGTA AGTTGAATAC TTAGCACCCA GGGGTAATCA GCTTGGACAG
    ACTCTTCTAT ATAAGACCAT TCAACTTATG AATCGTGGGT CCCCATTAGT CGAACCTGTC

841 GACCAGGTCC AAAGACTGTT AAGAGTCTTC TGACTCCAAA CTCAGTGCTC CCTCCAGTGC
    CTGGTCCAGG TTTCTGACAA TTCTCAGAAG ACTGAGGTTT GAGTCACGAG GGAGGTCACG

901 CACAAGCAAA CTCCATAAAG GTATCCTGTG CTGAATAGAG ACTGTAGAGT GGTACAAAGT
    GTGTTCGTTT GAGGTATTTC CATAGGACAC GACTTATCTC TGACATCTCA CCATGTTTCA

961 AAGACAGACA TTATATTAAG TCTTAGCTTT GTGACTTCGA ATGACTTACC TAATCTAGCT
    TTCTGTCTGT AATATAATTC AGAATCGAAA CACTGAAGCT TACTGAATGG ATTAGATCGA

1021 AAATTTCAGT TTTACCATGT GTAAATCAGG AAGAGTAATA GAACAAACCT TGAAGGGTCC
     TTTAAAGTCA AAATGGTACA CATTTAGTCC TTCTCATTAT CTTGTTTGGA ACTTCCCAGG

1081 CAATGGTGAT TAAATGAGGT GATGTACATA ACATGCATCA CTCATAATAA GTGCTCTTTA
     GTTACCACTA ATTTACTCCA CTACATGTAT TGTACGTAGT GAGTATTATT CACGAGAAAT

1141 AATATTAGTC ACTATTATTA GCCATCTCTG ATTAGATTTG ACAATAGGAA CATTAGGAAA
     TTATAATCAG TGATAATAAT CGGTAGAGAC TAATCTAAAC TGTTATCCTT GTAATCCTTT

1201 GATATAGTAC ATTCAGGATT TTGTTAGAAA GAGATGAAGA AATTCCCTTC CTTCCTGCCC
     CTATATCATG TAAGTCCTAA AACAATCTTT CTCTACTTCT TTAAGGGAAG GAAGGACGGG

1261 TAGGTCATCT AGGAGTTGTC ATGGTTCATT GTTGACAAAT TAATTTTCCC AAATTTTTCA
     ATCCAGTAGA TCCTCAACAG TACCAAGTAA CAACTGTTTA ATTAAAAGGG TTTAAAAAGT

1321 CTTTGCTCAG AAAGTCTACA TCGAAGCACC CAAGACTGTA CAATCTAGTC CATCTTTTTC
     GAAACGAGTC TTTCAGATGT AGCTTCGTGG GTTCTGACAT GTTAGATCAG GTAGAAAAAG

1381 CACTTAACTC ATACTGTGCT CTCCCTTTCT CAAAGCAAAC TGTTTGCTAT TCCTTGAATA
     GTGAATTGAG TATGACACGA GAGGGAAAGA GTTTCGTTTG ACAAACGATA AGGAACTTAT

1441 CACTCTGAGT TTTCTGCCTT TGCCTACTCA GCTGGCCCAT GGCCCCTAAT GTTTCTTCTC
     GTGAGACTCA AAAGACGGAA ACGGATGAGT CGACCGGGTA CCGGGGATTA CAAAGAAGAG

1501 ATCTCCACTG GGTCAAATCC TACCTGTACC TTATGGTTCT GTTAAAAGCA GTGCTTCCAT
     TAGAGGTGAC CCAGTTTAGG ATGGACATGG AATACCAAGA CAATTTTCGT CACGAAGGTA

1561 AAAGTACTCC TAGCAAATGC ACGGCCTCTC TCACGGATTA TAAGAACACA GTTTATTTTA
     TTTCATGAGG ATCGTTTACG TGCCGGAGAG AGTGCCTAAT ATTCTTGTGT CAAATAAAAT

1621 TAAAGCATGT AGCTATTCTC TCCCTCGAAA TACGATTATT ATTATTAAGA ATTTATAGCA
     ATTTCGTACA TCGATAAGAG AGGGAGCTTT ATGCTAATAA TAATAATTCT TAAATATCGT

1681 GGGATATAAT TTTGTATGAT GATTCTTCTG GTTAATCCAA CCAAGATTGA TTTTATATCT
     CCCTATATTA AAACATACTA CTAAGAAGAC CAATTAGGTT GGTTCTAACT AAAATATAGA

1741 ATTACGTAAG ACAGTAGCCA GACATAGCCG GGATATGAAA ATAAAGTCTC TGCCTTCAAC
     TAATGCATTC TGTCATCGGT CTGTATCGGC CCTATACTTT TATTTCAGAG ACGGAAGTTG

1801 AAGTTCCAGT ATTCTTTTCT TTCCTCCCCT CCCCTCCCCT CCCTTCCCCT CCCCTTCCTT
     TTCAAGGTCA TAAGAAAAGA AAGGAGGGGA GGGGAGGGGA GGGAAGGGGA GGGGAAGGAA

1861 CCCTTTCCCT TCCCTTCCTT TCTTTCTTGA GGGAGTCTCA CTCTGTCACC AGGCTCCAGT
     GGGAAAGGGA AGGGAAGGAA AGAAAGAACT CCCTCAGAGT GAGACAGTGG TCCGAGGTCA
```

FIGURE 15C

```
1921 GCAGTGGCGC TATCTTGGCT GACTGCAACC TCCGCCTCCC CGGTTCAAGC GATTCTCCTG
     CGTCACCGCG ATAGAACCGA CTGACGTTGG AGGCGGAGGG GCCAAGTTCG CTAAGAGGAC

1981 CCTCAGCCTC CTGAGTAGCT GGGACTACAG GAGCCCGCCA CCACGCCCAG CTAATTTTTG
     GGAGTCGGAG GACTCATCGA CCCTGATGTC CTCGGGCGGT GGTGCGGGTC GATTAAAAAC

2041 TATTTTTAGT AGAGATGGGG TTTCACCATG TTGGCCAGGA TGGTCTCGAT CTCTCGACTT
     ATAAAAATCA TCTCTACCCC AAAGTGGTAC AACCGGTCCT ACCAGAGCTA GAGAGCTGAA

2101 CGTGATCCGC CTGTCTGGGC CTCCCAAAGT GCTGGGATTA CAGGCGTGAG CCACCACGCC
     GCACTAGGCG GACAGACCCG GAGGGTTTCA CGACCCTAAT GTCCGCACTC GGTGGTGCGG

2161 CGGCTTTAAA AAATGGTTTT GTAATGTAAG TGGAGGATAA TACCCTACAT GTTTATTAAT
     GCCGAAATTT TTTACCAAAA CATTACATTC ACCTCCTATT ATGGGATGTA CAAATAATTA

2221 AACAATAATA TTCTTTAGGA AAAAGGGCGC GGTGGTGATT TACACTGATG ACAAGCATTC
     TTGTTATTAT AAGAAATCCT TTTTCCCGCG CCACCACTAA ATGTGACTAC TGTTCGTAAG

2281 CCGACTATGG AAAAAAAGCG CAGCTTTTTC TGCTCTGCTT TTATTCAGTA GAGTATTGTA
     GGCTGATACC TTTTTTTCGC GTCGAAAAAG ACGAGACGAA AATAAGTCAT CTCATAACAT

2341 GAGATTGTAT AGAATTTCAG AGTTGAATAA AAGTTCCTCA TAATTATAGG AGTGGAGAGA
     CTCTAACATA TCTTAAAGTC TCAACTTATT TTCAAGGAGT ATTAATATCC TCACCTCTCT

2401 GGAGAGTCTC TTTCTTCCTT TCATTTTTAT ATTTAAGCAA GAGCTGGACA TTTTCCAAGA
     CCTCTCAGAG AAAGAAGGAA AGTAAAAATA TAAATTCGTT CTCGACCTGT AAAAGGTTCT

2461 AAGTTTTTTT TTTTTAAGGC GCCTCTCAAA AGGGGCCGGA TTTCCTTCTC CTGGAGGCAG
     TTCAAAAAAA AAAAATTCCG CGGAGAGTTT TCCCCGGCCT AAAGGAAGAG GACCTCCGTC

2521 ATGTTGCCTC TCTCTCTCGC TCGGATTGGT TCAGTGCACT CTAGAAACAC TGCTGTGGTG
     TACAACGGAG AGAGAGAGCG AGCCTAACCA AGTCACGTGA GATCTTTGTG ACGACACCAC

2581 GAGAAACTGG ACCCCAGGTC TGGAGCGAAT TCCAGCCTGC AGGGCTGATA AGCGAGGCAT
     CTCTTTGACC TGGGGTCCAG ACCTCGCTTA AGGTCGGACG TCCCGACTAT TCGCTCCGTA

2641 TAGTGAGATT GAGAGAGACT TTACCCCGCC GTGGTGGTTG GAGGGCGCGC AGTAGAGCAG
     ATCACTCTAA CTCTCTCTGA AATGGGGCGG CACCACCAAC CTCCCGCGCG TCATCTCGTC

2701 CAGCACAGGC GCGGGTCCCG GGAGGCCGGC TCTGCTCGCG CCGAGATGTG GAATCTCCTT
     GTCGTGTCCG CGCCCAGGGC CCTCCGGCCG AGACGAGCGC GGCTCTACAC CTTAGAGGAA

2761 CACGAAACCG ACTCGGCTGT GGCCACCGCG CGCCGCCCGC GCTGGCTGTG CGCTGGGGCG
     GTGCTTTGGC TGAGCCGACA CCGGTGGCGC GCGGCGGGCG CGACCGACAC GCGACCCCGC

2821 CTGGTGCTGG CGGGTGGCTT CTTTCTCCTC GGCTTCCTCT TCGGTAGGGG GGCGCCTCGC
     GACCACGACC GCCCACCGAA GAAAGAGGAG CCGAAGGAGA AGCCATCCCC CCGCGGAGCG

2881 GGAGCAAACC TCGGAGTCTT CCCCGTGGTG CCGCGGTGCT GGGACTCGCG GGTCAGCTGC
     CCTCGTTTGG AGCCTCAGAA GGGGCACCAC GGCGCCACGA CCCTGAGCGC CCAGTCGACG

2941 CGAGTGGGAT CCTGTTGCTG GTCTTCCCCA GGGGCGGCGA TTAGGGTCGG GGTAATGTGG
     GCTCACCCTA GGACAACGAC CAGAAGGGGT CCCCGCCGCT AATCCCAGCC CCATTACACC

3001 GGTGAGCACC CCTCGAG
     CCACTCGTGG GGAGCTC
```

FIGURE 15D

```
- 2401 GGAGAGTCTC TTTCTTCCTT TCATTTTTAT ATTTAAGCAA GAGCTGGACA TTTTCCAAGA
       CCTCTCAGAG AAAGAAGGAA AGTAAAAATA TAAATTCGTT CTCGACCTGT AAAAGGTTCT

- 2461 AAGTTTTTTT TTTTTAAGGC GCCTCTCAAA AGGGGCCGGA TTTCCTTCTC CTGGAGGCAG
       TTCAAAAAAA AAAAATTCCG CGGAGAGTTT TCCCCGGCCT AAAGGAAGAG GACCTCCGTC

- 2521 ATGTTGCCTC TCTCTCTCGC TCGGATTGGT TCAGTGCACT CTAGAAACAC TGCTGTGGTG
       TACAACGGAG AGAGAGAGCG AGCCTAACCA AGTCACGTGA GATCTTTGTG ACGACACCAC

- 2581 GAGAAACTGG ACCCCAGGTC TGGAGCGAAT TCCAGCCTGC AGGGCTGATA AGCGAGGCAT
       CTCTTTGACC TGGGGTCCAG ACCTCGCTTA AGGTCGGACG TCCCGACTAT TCGCTCCGTA

- 2641 TAGTGAGATT GAGAGAGACT TTACCCCGCC GTGGTGGTTG GAGGGCGCGC AGTAGAGCAG
       ATCACTCTAA CTCTCTCTGA AATGGGGCGG CACCACCAAC CTCCCGCGCG TCATCTCGTC

- 2701 CAGCACAGGC GCGGGTCCCG GGAGGCCGGC TCTGCTCGCG CCGAGATGTG GAATCTCCTT
       GTCGTGTCCG CGCCCAGGGC CCTCCGGCCG AGACGAGCGC GGCTCTACAC CTTAGAGGAA

- 2761 CACGAAACCG ACTCGGCTGT GGCCACCGCG CGCCGCCCGC GCTGGCTGTG CGCTGGGGCG
       GTGCTTTGGC TGAGCCGACA CCGGTGGCGC GCGGCGGGCG CGACCGACAC GCGACCCCGC

- 2821 CTGGTGCTGG CGGGTGGCTT CTTTCTCCTC GGCTTCCTCT TCGGTAGGGG GGCGCCTCGC
       GACCACGACC GCCCACCGAA GAAAGAGGAG CCGAAGGAGA AGCCATCCCC CCGCGGAGCG

- 2881 GGAGCAAACC TCGGAGTCTT CCCCGTGGTG CCGCGGTGCT GGGACTCGCG GGTCAGCTGC
       CCTCGTTTGG AGCCTCAGAA GGGGCACCAC GGCGCCACGA CCCTGAGCGC CCAGTCGACG

- 2941 CGAGTGGGAT CCTGTTGCTG GTCTTCCCCA GGGGCGGCGA TTAGGGTCGG GGTAATGTGG
       GCTCACCCTA GGACAACGAC CAGAAGGGGT CCCCGCCGCT AATCCCAGCC CCATTACACC

- 3001 GGTGAGCACC CCTCGAG
       CCACTCGTGG GGAGCTC
```

FIGURE 16

Potential binding sites on the PSM promoter*

Site	Seq	**Location	#nt matched	
AP1	TKAGTCA	-1145	7/7	
E2-RS	ACCNNNNNNGGT	-1940	12/12	
		-1951	12/12	
GHF	NNNTAAATNNN	-580	11/11	
		-753	11/11	
		-1340	11/11	
		-1882	11/11	
		-1930	11/11	
		-1979	11/11	
		-2001	11/11	
		-2334	11/11	
		-2374	11/11	
		-2591	11/11	
		-2620	11/11	
		-2686	11/11	
JVC repeat	GGGNGGRR	-1165	8/8	
		-1175	8/8	
		-1180	8/8	
		-1185	8/8	
		-1190	8/8	
NFkB	GGGRHTYYHC	-961	10/10	
uteroglobi	RYYWSGTG	-250	8/8	
		-921	8/8	
		-1104	8/8	
IFN	AAWAANGAAAGGR	590	13/13	Cell 41:509 (1985)

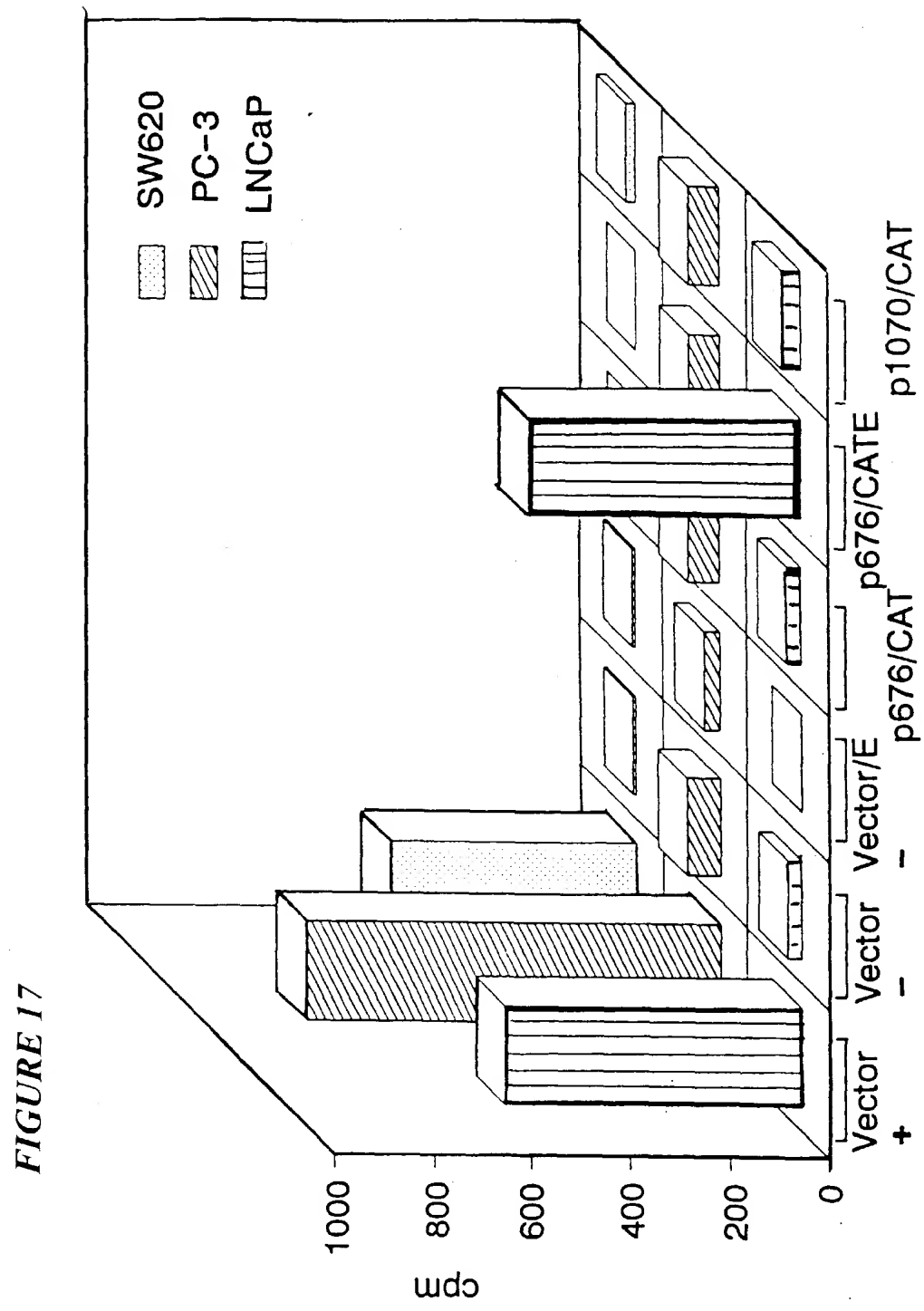

SW620
PC-3
LNCaP
cpm
1000
800
600
400
200
0
+
–
–
Vector
Vector
Vector/E
p676/CAT
p676/CATE
p1070/CAT

FIGURE 17

FIGURE 18

```
                                                                CTCAAAAGGGGCCGGATTTCCT
TCT TGGAGGCAGATGTTGCCTCTCTCTCTCGCTCGGATTGGTTCAGTGCACTCTAGAAACACTGCTGTGGTGGAGAAACT
GGACCCC AGG TCTGGAGCGAATTCCA GCCTGCAGGGCTGATAAGCGAGGCATTAGTGAGATTGAGAGAGACTTTACCC
CGCGGTGGTTGGAGGGCGCGCAGT AGAGCAGCAGCACAGGCGCGGGTCCCGGGAGGCCGGCTCTGCTCGCGCCGAG

ATG TGG AAT CTC CTT CAC GAA ACC GAC TCG GCT GTG GCC ACC GCG CGC CGC CCG CGC TGG CTG
Met Trp Asn Leu Leu His Glu Thr Asp Ser Ala Val Ala Ala Ala Arg Arg Pro Arg Trp Leu

TGC GCT GGG GCG CTG GTG CTG GCG GGT GGC TTC TTT CTC CTC GGC TTC CTC TTC GGA TGG TTT
Cys Ala Gly Ala Leu Val Leu Ala Gly Gly Phe Phe Leu Leu Gly Phe Leu Phe Gly Trp Phe

ATA AAA TCC TCC AAT GAA GCT ACT AAC ATT ACT CCA AAG CAT AAT ATG AAA GCA TTT TTG GAT GAA
Ile Lys Ser Ser Asn Glu Ala Thr Asn Ile Thr Pro Lys His Asn Met Lys Ala Phe Leu Asp Glu
                                                    *

TGG AAA GCT GAG AAC ATC AAG AAG TTC TTA TAT AAT TTT ACA CAG ATA CCA CAT TTA GCA GGA ACA
Leu Lys Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile Pro His Leu Ala Gly Thr
```

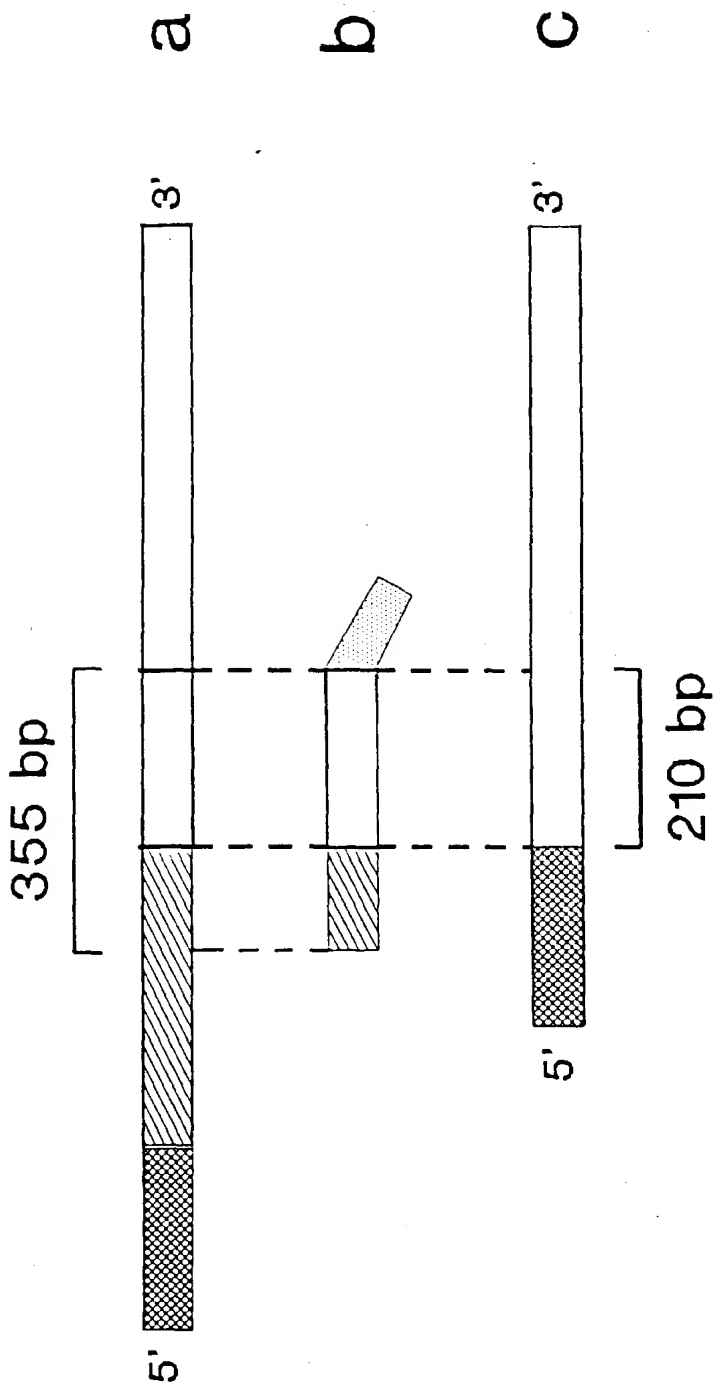


FIGURE 19

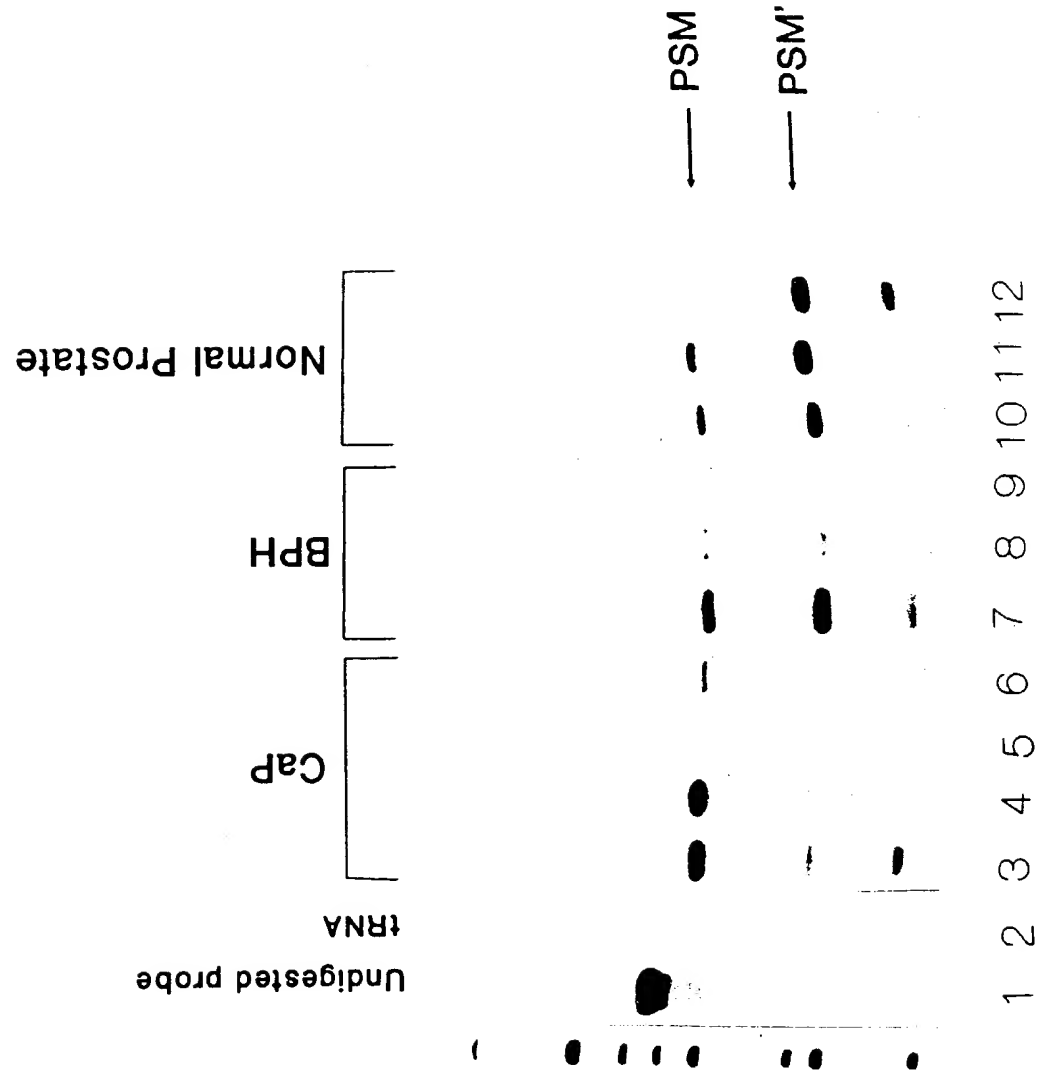

PSM
PSM'
Normal Prostate
BPH
CaP
tRNA
Undigested probe
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 20

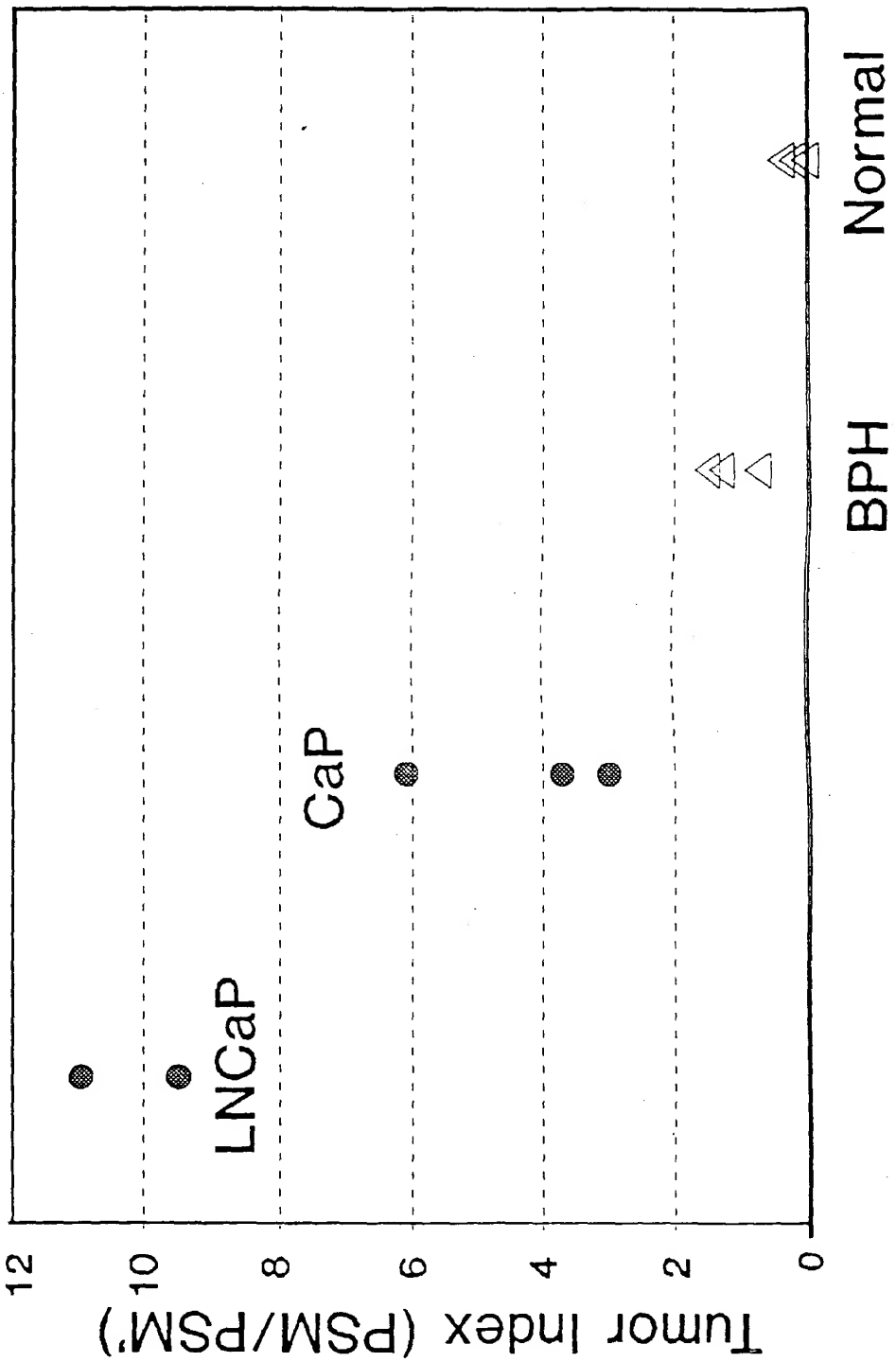


FIGURE 21

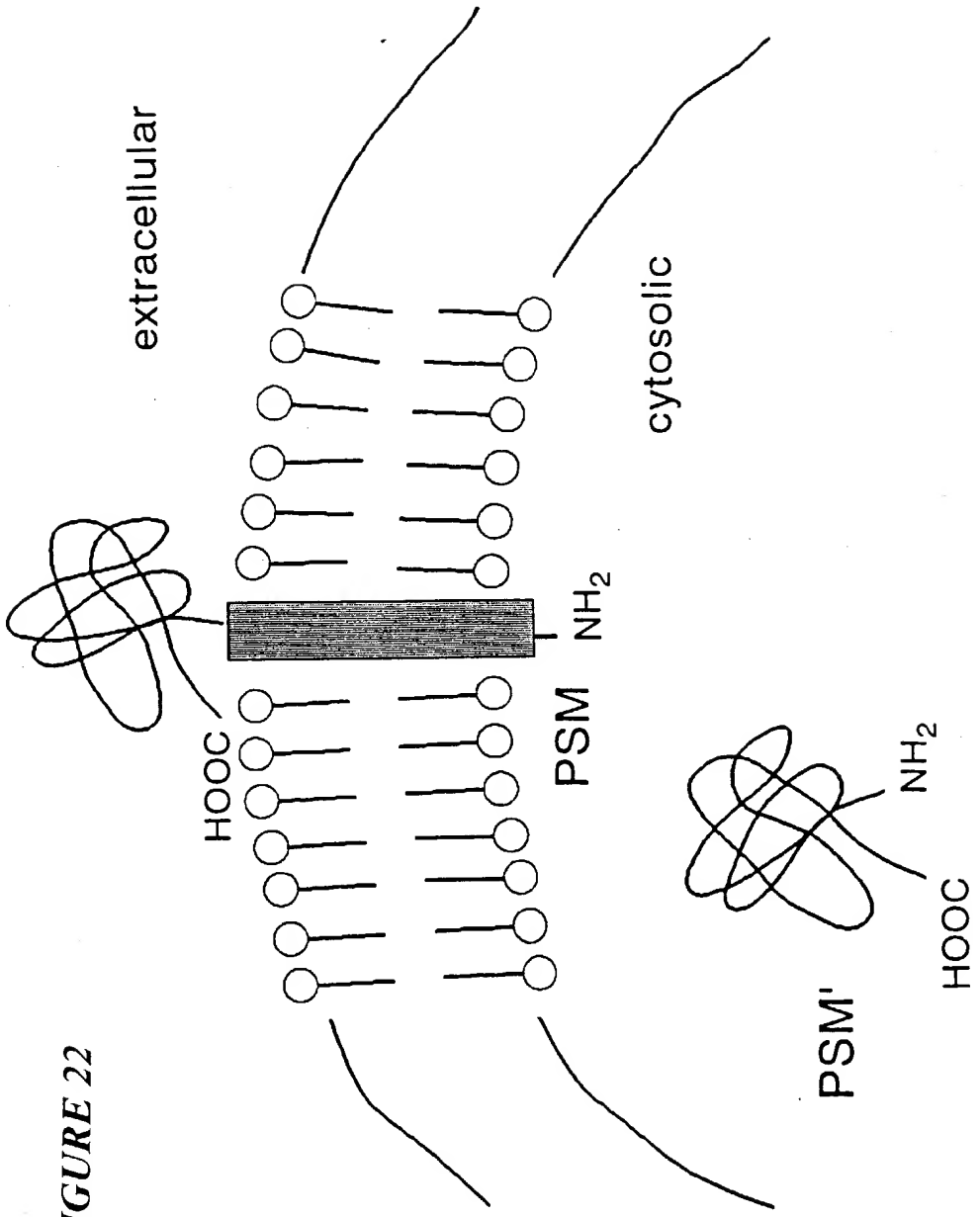
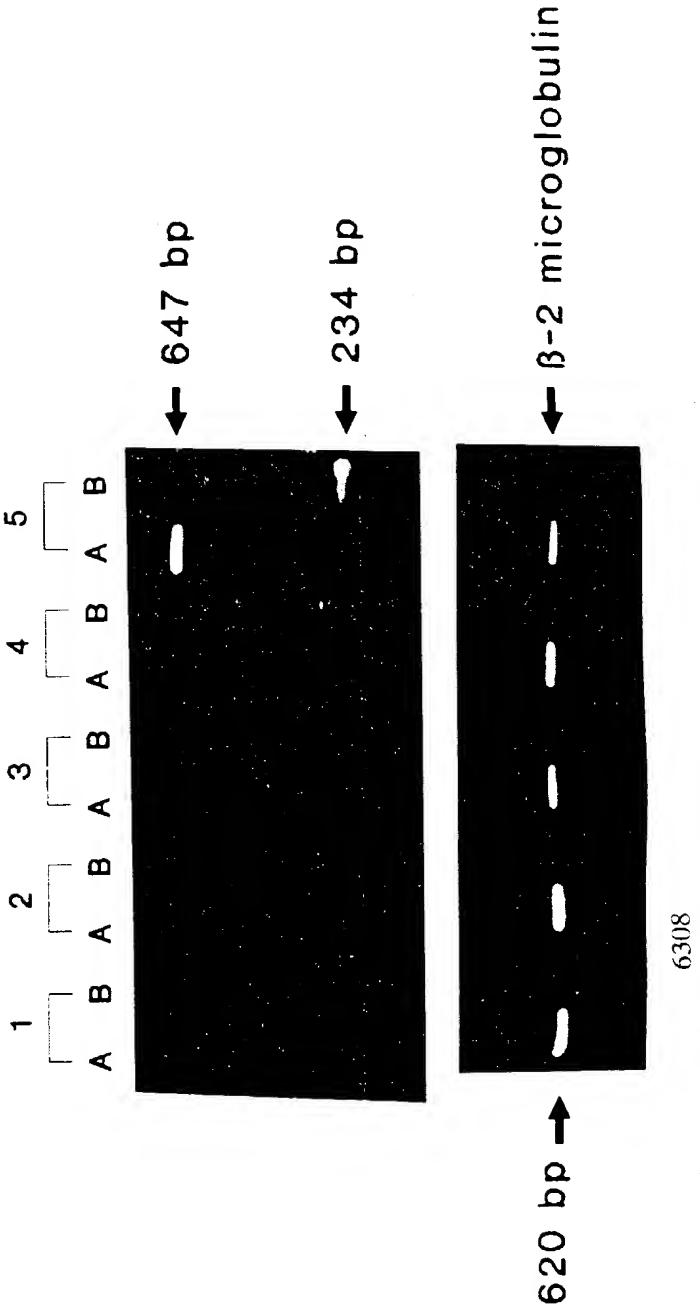


FIGURE 22

FIGURE 23



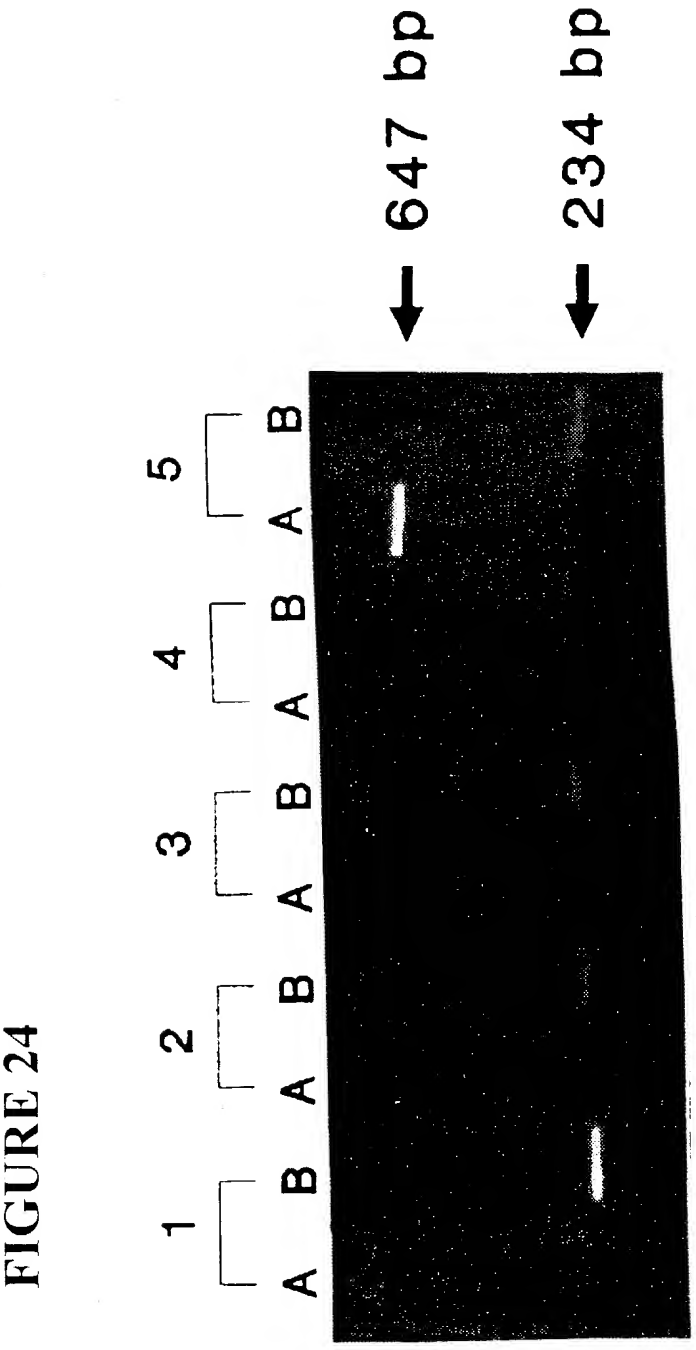


FIGURE 24

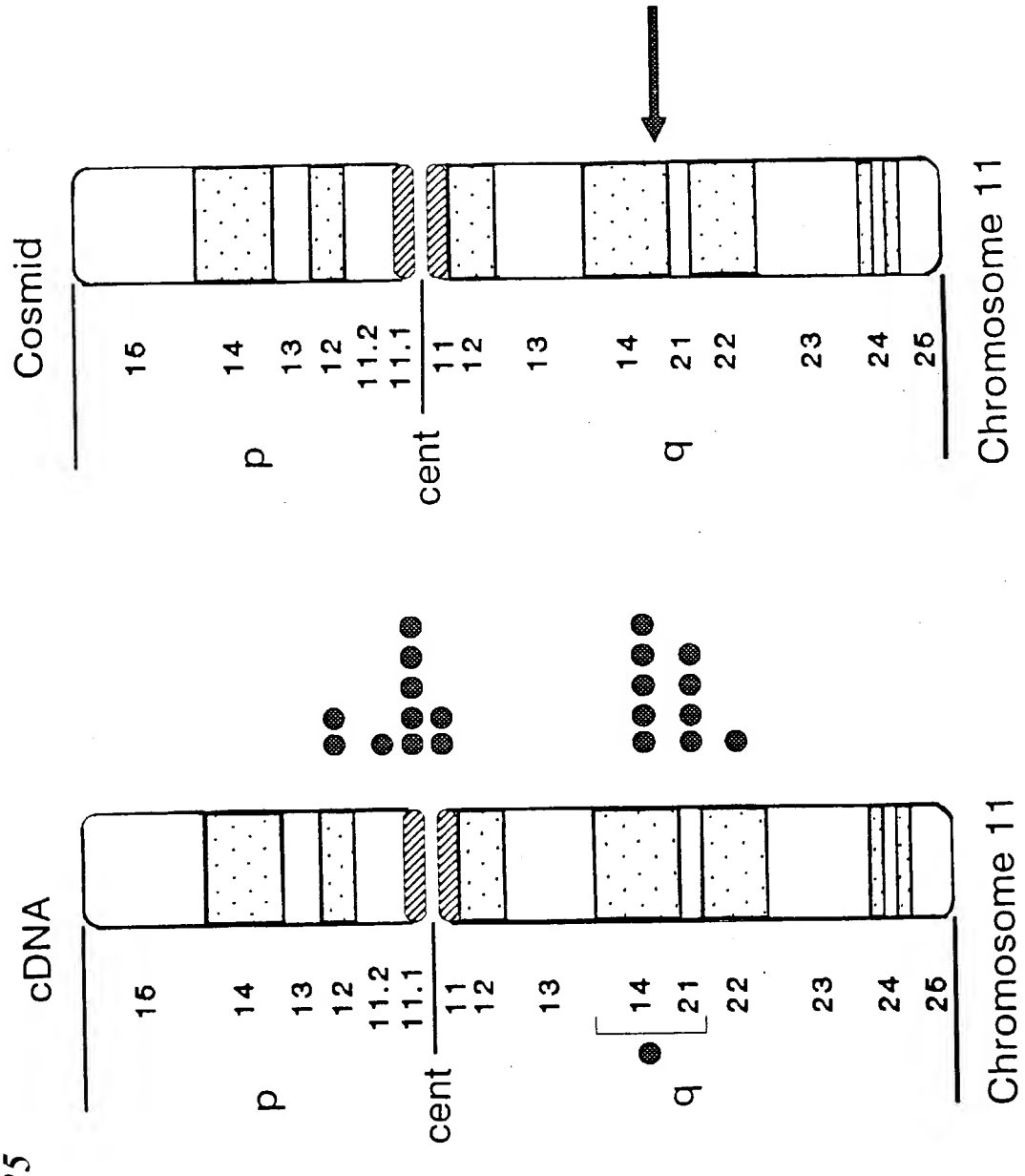

Cosmid
cDNA
16
14
13
12
11.2
11.1
11
12
13
14
21
22
23
24
25
p
cent
q
Chromosome 11

FIGURE 25

FIGURE 26

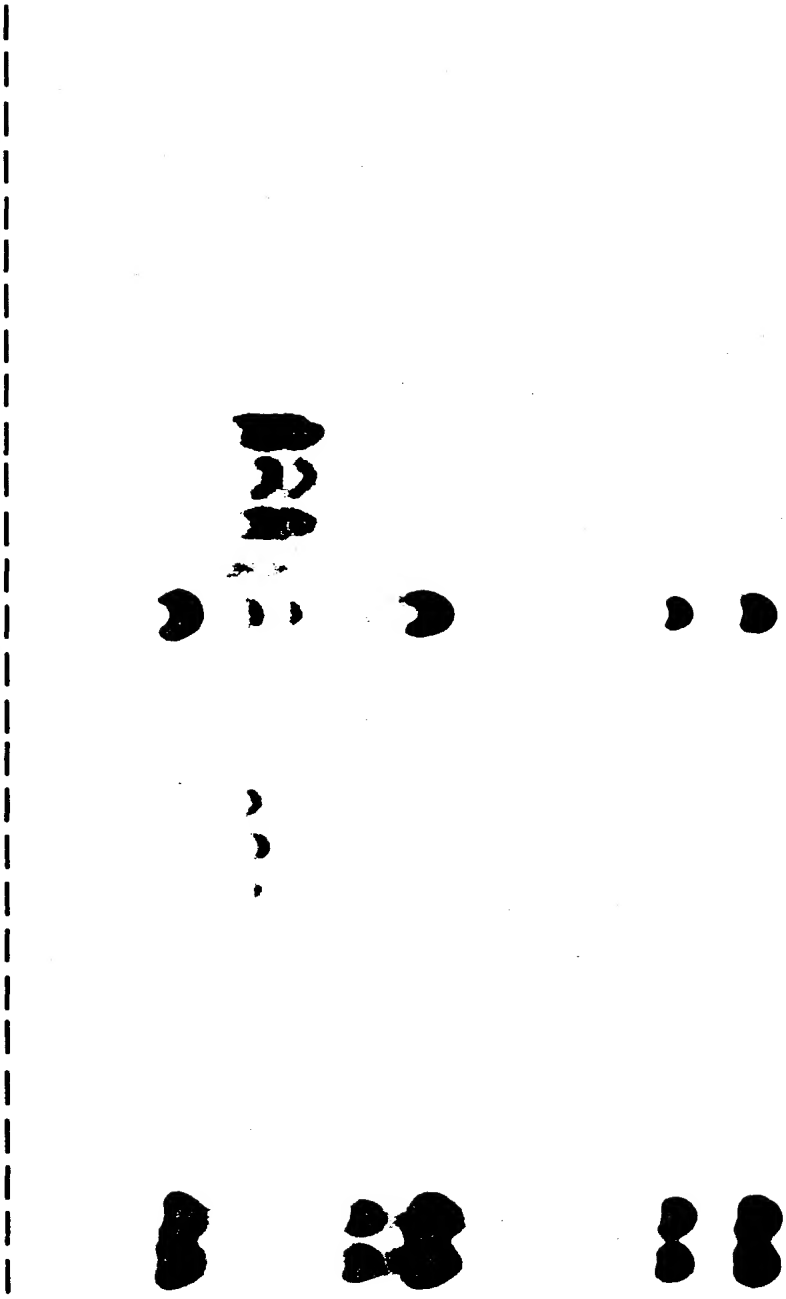
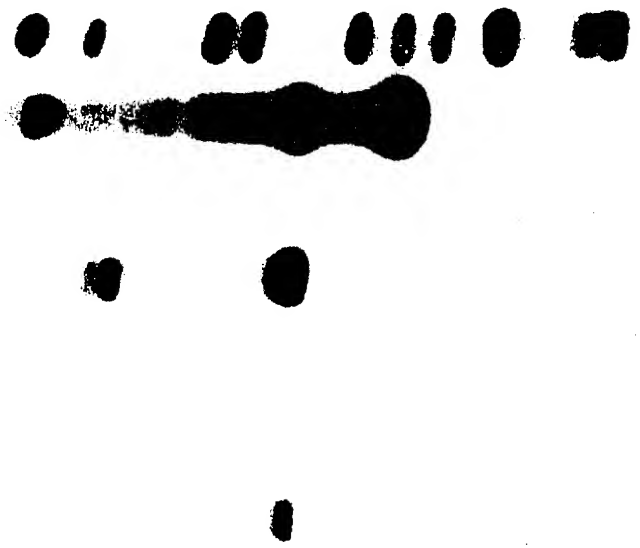


FIGURE 27



Markers

Uncut

t RNA

LnCap

PC 3

AT6.1

AT6.1-11 clone 1

AT6.1-11 clone 2

A9

A9 (11)

R1564

R1564-11 clone 4

R1564-11 clone 6

FIGURE 28

TISSUE/ CELL LINE	CANCER CELL TYPE	[1]PSM DNA	[2]PSM RNA
HUMAN PROSTATE	N.A.	+	+
HUMAN MAMMARY	N.A.	+	-
AT6.1	RAT PROSTATIC ADENOCARCINOMA	-	-
AT6.1-11-CL1	"	+	+
AT6.1-11-CL2	"	-	-
R1564	RAT MAMMARY ADENOCARCINOMA	-	-
R1564-11-CL2	"	+	-
R1564-11-CL4	"	+	-
R1564-11-CL5	"	+	-
R1564-11-CL6	"	+	-
A9	MOUSE FIBROSARCOMA	-	-
A9(11)	"	+	-

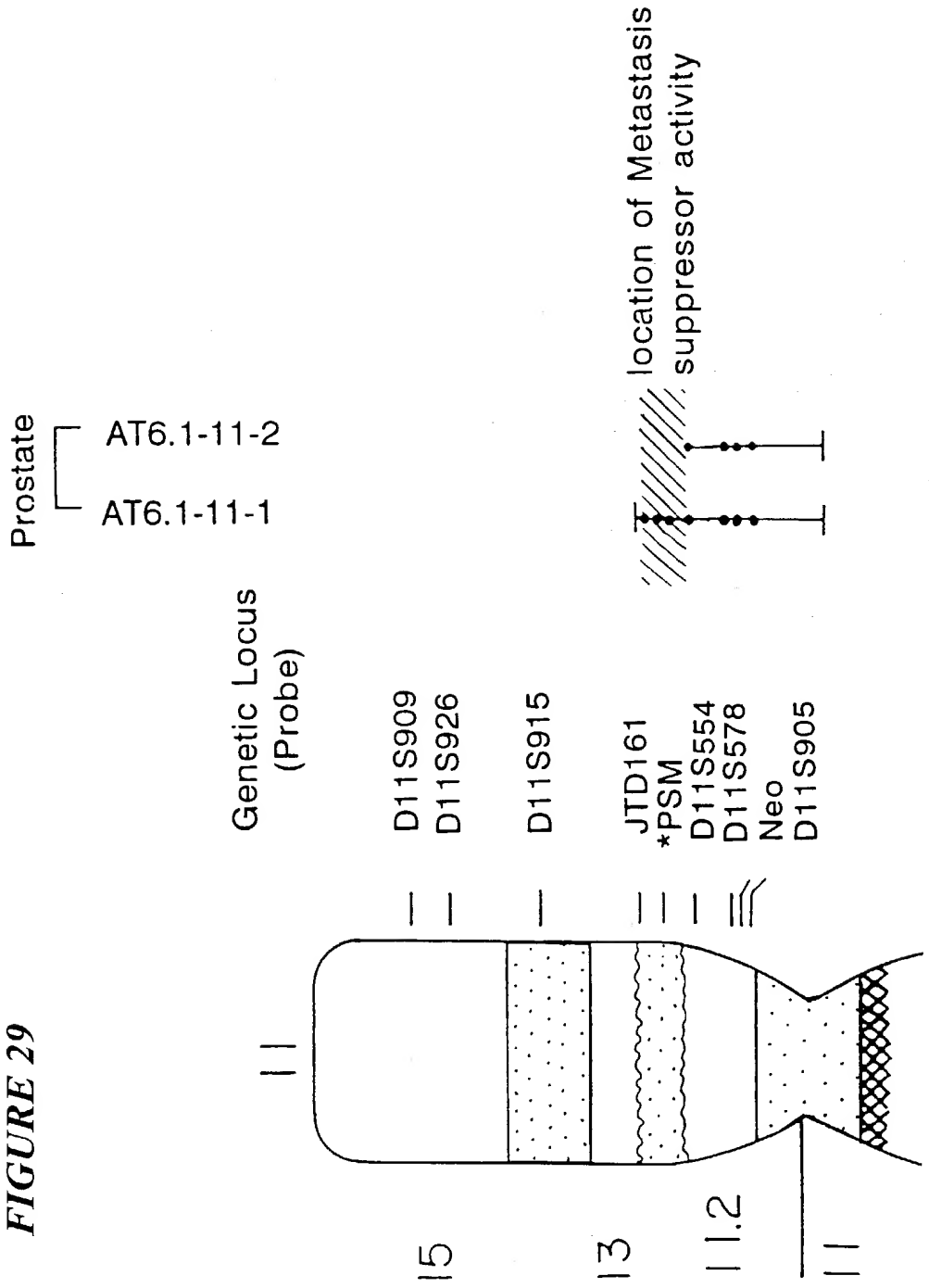

Prostate
AT6.1-11-2
AT6.1-11-1
location of Metastasis suppressor activity
Genetic Locus (Probe)
D11S909
D11S926
D11S915
JTD161
*PSM
D11S554
D11S578
Neo
D11S905
11
15
13
11.2
11

FIGURE 29

FIGURE 30

FIG. 55

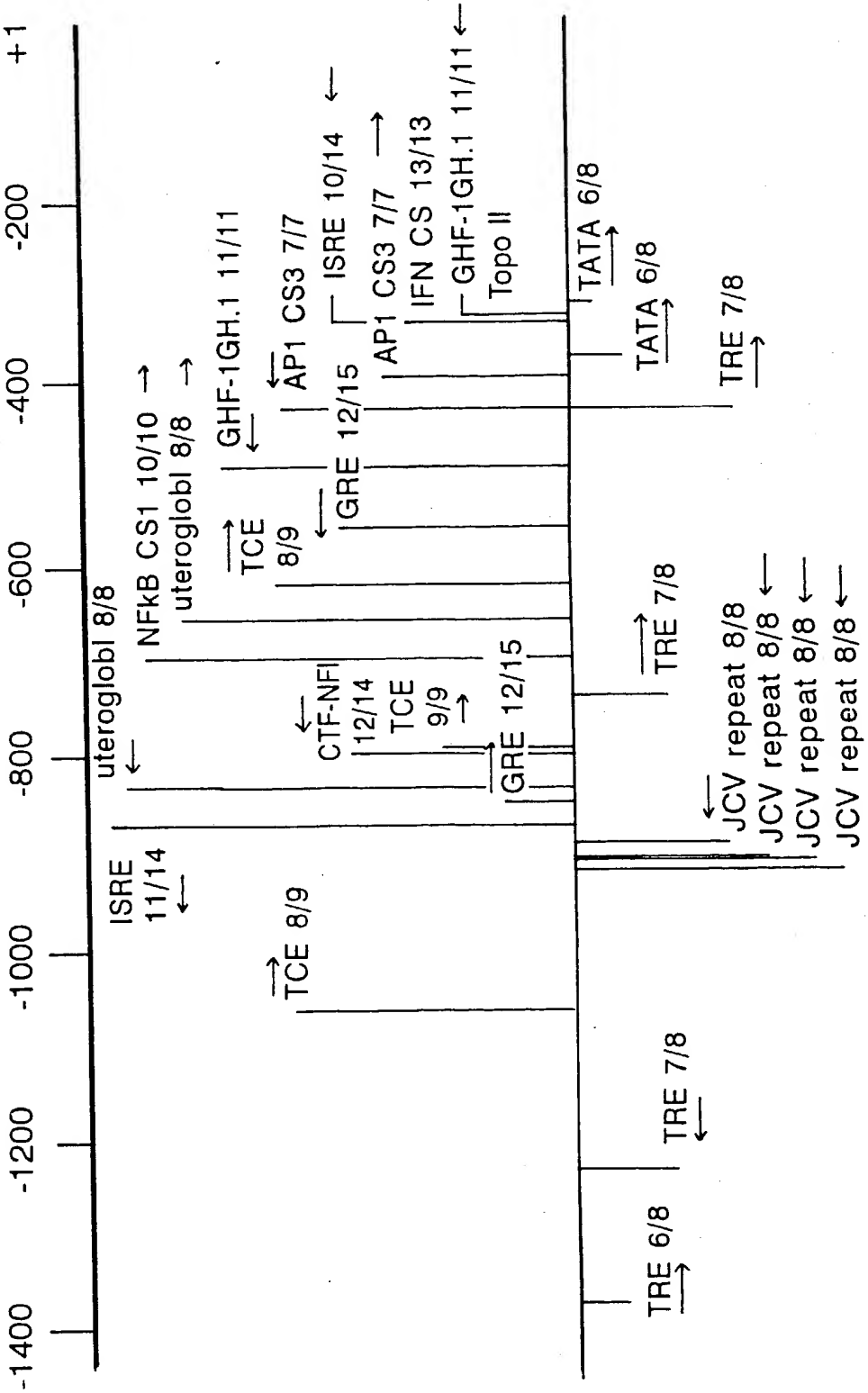


FIGURE 31

Prostate Specific Promoter: Cytosine Deaminase Chimera

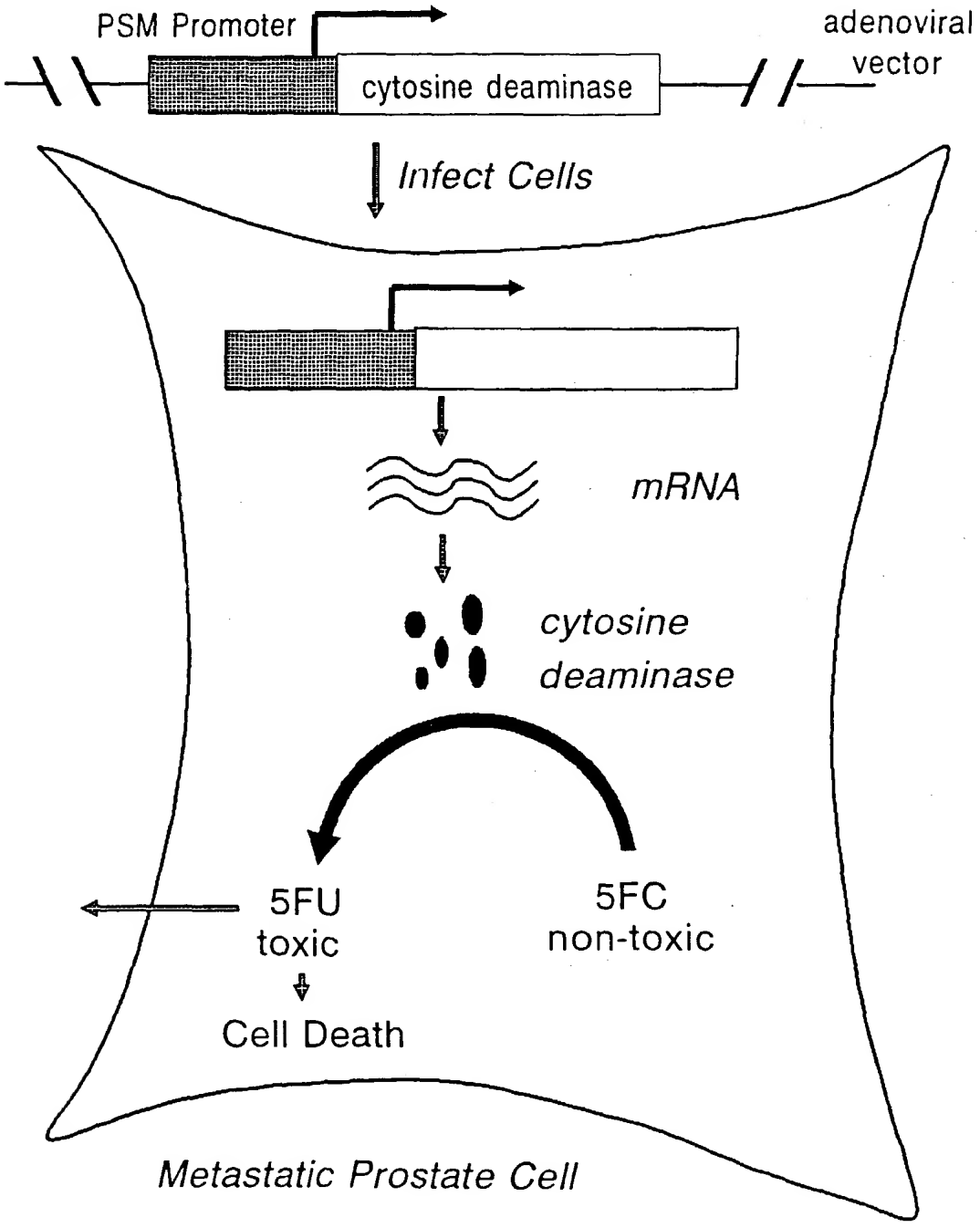


FIGURE 32A

```
          10         20         30         40         50         60
          |          |          |          |          |          |
1 AAGGGTGCTC CTTAGGCTGA ATGCTTGCAG ACAGGATGCT TGGTTACAGA TGGGCTGTGA
  TTCCCACGAG GAATCCGACT TACGAACGTC TGTCCTACGA ACCAATGTCT ACCCGACACT

61 CTCGAGTGGA GTTTTATAAG GGTGCTCCTT AGGCTGAATG CTTGCAGACA GGATGCTTGG
   GAGCTCACCT CAAAATATTC CCACGAGGAA TCCGACTTAC GAACGTCTGT CCTACGAACC

121 TTACAGATGG GCTGTGAGCT GGGTGCTTGT AAGAGGATGC TTGGGTGCTA AGTGAGCCAT
    AATGTCTACC CGACACTCGA CCCACGAACA TTCTCCTACG AACCCACGAT TCACTCGGTA

181 TTGCAGTTGA CCCTATTCTT GGAACATTCA TTCCCCTCTA CCCCTGTTTC TGTTCCTGCC
    AACGTCAACT GGGATAAGAA CCTTGTAAGT AAGGGGAGAT GGGGACAAAG ACAAGGACGG

241 AGCTAAGCCC ATTTTTCATT TTTCTTTTAA CTCCTTAGCG CTCCGCAAAA CTTAATCAAT
    TCGATTCGGG TAAAAAGTAA AAAGAAAATT GAGGAATCGC GAGGCGTTTT GAATTAGTTA

301 TTCTTTAAAC CTCAGTTTTC TTATCTGTAA AAGGTAAATA ATAATACAGG GTGCAACAGA
    AAGAAATTTG GAGTCAAAAG AATAGACATT TTCCATTTAT TATTATGTCC CACGTTGTCT

361 AAAATCTAGT GTGGTTTACA TAATCACCTG TTAGAGATTT TAAATTATTT CAGGATAAGT
    TTTTAGATCA CACCAAATGT ATTAGTGGAC AATCTCTAAA ATTTAATAAA GTCCTATTCA

421 CATGATAATT AAATGAAATA ATGCACATAA AGCACATAGT GTGGTGTCCT CCATATAGAA
    GTACTATTAA TTTACTTTAT TACGTGTATT TCGTGTATCA CACCACAGGA GGTATATCTT

481 AATGCTCAGT ATATTGGTTA TTAACTACTT GTTGAAGGTT TATCTTCTCC ACTAAACTGT
    TTACGAGTCA TATAACCAAT AATTGATGAA CAACTTCCAA ATAGAAGAGG TGATTTGACA

541 AAGTTCCACA AGCCTTACAA TATGTGACAG ATATTCATTC ATTGTCTGAA TTCTTCAAAT
    TTCAAGGTGT TCGGAATGTT ATACACTGTC TATAAGTAAG TAACAGACTT AAGAAGTTTA

601 ACATCCTCTT CACCATAGCG TCTTATTAAT TGAATTATTA ATTGAATAAA TTCTATTGTT
    TGTAGGAGAA GTGGTATCGC AGAATAATTA ACTTAATAAT TAACTTATTT AAGATAACAA

661 CAAAAATCAC TTTTATATTT AACTGAAATT TGCTTACTTA TAATCACATC TAACCTTCAA
    GTTTTTAGTG AAAATATAAA TTGACTTTAA ACGAATGAAT ATTAGTGTAG ATTGGAAGTT

721 AGAAAACACA TTAACCAACT GTACTGGGTA ATGTTACTGG GTGATCCCAC GTTTTACAAA
    TCTTTTGTGT AATTGGTTGA CATGACCCAT TACAATGACC CACTAGGGTG CAAAATGTTT
```

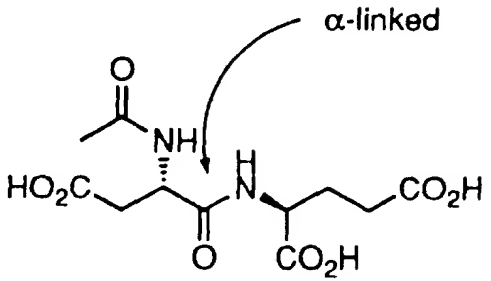
FIGURE 32B

```
 781 TGAGAAGATA TATTCTGGTA AGTTGAATAC TTAGCACCCA GGGGTAATCA GCTTGGACAG
     ACTCTTCTAT ATAAGACCAT TCAACTTATG AATCGTGGGT CCCCATTAGT CGAACCTGTC

 841 GACCAGGTCC AAAGACTGTT AAGAGTCTTC TGACTCCAAA CTCAGTGCTC CCTCCAGTGC
     CTGGTCCAGG TTTCTGACAA TTCTCAGAAG ACTGAGGTTT GAGTCACGAG GGAGGTCACG

 901 CACAAGCAAA CTCCATAAAG GTATCCTGTG CTGAATAGAG ACTGTAGAGT GGTACAAAGT
     GTGTTCGTTT GAGGTATTTC CATAGGACAC GACTTATCTC TGACATCTCA CCATGTTTCA

 961 AAGACAGACA TTATATTAAG TCTTAGCTTT GTGACTTCGA ATGACTTACC TAATCTAGCT
     TTCTGTCTGT AATATAATTC AGAATCGAAA CACTGAAGCT TACTGAATGG ATTAGATCGA

1021 AAATTTCAGT TTTACCATGT GTAAATCAGG AAGAGTAATA GAACAAACCT TGAAGGGTCC
     TTTAAAGTCA AAATGGTACA CATTTAGTCC TTCTCATTAT CTTGTTTGGA ACTTCCCAGG

1081 CAATGGTGAT TAAATGAGGT GATGTACATA ACATGCATCA CTCATAATAA GTGCTCTTTA
     GTTACCACTA ATTTACTCCA CTACATGTAT TGTACGTAGT GAGTATTATT CACGAGAAAT

1141 AATATTAGTC ACTATTATTA GCCATCTCTG ATTAGATTTG ACAATAGGAA CATTAGGAAA
     TTATAATCAG TGATAATAAT CGGTAGAGAC TAATCTAAAC TGTTATCCTT GTAATCCTTT

1201 GATATAGTAC ATTCAGGATT TTGTTAGAAA GAGATGAAGA AATTCCCTTC CTTCCTGCCC
     CTATATCATG TAAGTCCTAA AACAATCTTT CTCTACTTCT TTAAGGGAAG GAAGGACGGG

1261 TAGGTCATCT AGGAGTTGTC ATGGTTCATT GTTGACAAAT TAATTTTCCC AAATTTTTCA
     ATCCAGTAGA TCCTCAACAG TACCAAGTAA CAACTGTTTA ATTAAAAGGG TTTAAAAAGT

1321 CTTTGCTCAG AAAGTCTACA TCGAAGCACC CAAGACTGTA CAATCTAGTC CATCTTTTTC
     GAAACGAGTC TTTCAGATGT AGCTTCGTGG GTTCTGACAT GTTAGATCAG GTAGAAAAAG

1381 CACTTAACTC ATACTGTGCT CTCCCTTTCT CAAAGCAAAC TGTTTGCTAT TCCTTGAATA
     GTGAATTGAG TATGACACGA GAGGGAAAGA GTTTCGTTTG ACAAACGATA AGGAACTTAT

1441 CACTCTGAGT TTTCTGCCTT TGCCTACTCA GCTGGCCCAT GGCCCCTAAT GTTTCTTCTC
     GTGAGACTCA AAAGACGGAA ACGGATGAGT CGACCGGGTA CCGGGGATTA CAAAGAAGAG

1501 ATCTCCACTG GGTCAAATCC TACCTGTACC TTATGGTTCT GTTAAAAGCA GTGCTTCCAT
     TAGAGGTGAC CCAGTTTAGG ATGGACATGG AATACCAAGA CAATTTTCGT CACGAAGGTA

1561 AAAGTACTCC TAGCAAATGC ACGGCCTCTC TCACGGATTA TAAGAACACA GTTTATTTTA
```

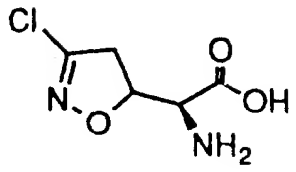
FIGURE 32C

```
     TTTCATGAGG ATCGTTTACG TGCCGGAGAG AGTGCCTAAT ATTCTTGTGT CAAATAAAAT

1621 TAAAGCATGT AGCTATTCTC TCCCTCGAAA TACGATTATT ATTATTAAGA ATTTATAGCA
     ATTTCGTACA TCGATAAGAG AGGGAGCTTT ATGCTAATAA TAATAATTCT TAAATATCGT

1681 GGGATATAAT TTTGTATGAT GATTCTTCTG GTTAATCCAA CCAAGATTGA TTTTATATCT
     CCCTATATTA AAACATACTA CTAAGAAGAC CAATTAGGTT GGTTCTAACT AAAATATAGA

1741 ATTACGTAAG ACAGTAGCCA GACATAGCCG GGATATGAAA ATAAAGTCTC TGCCTTCAAC
     TAATGCATTC TGTCATCGGT CTGTATCGGC CCTATACTTT TATTTCAGAG ACGGAAGTTG

1801 AAGTTCCAGT ATTCTTTTCT TTCCTCCCCT CCCCTCCCCT CCCTTCCCCT CCCCTTCCTT
     TTCAAGGTCA TAAGAAAAGA AAGGAGGGGA GGGGAGGGGA GGGAAGGGGA GGGGAAGGAA

1861 CCCTTTCCCT TCCCTTCCTT TCTTTCTTGA GGGAGTCTCA CTCTGTCACC AGGCTCCAGT
     GGGAAAGGGA AGGGAAGGAA AGAAAGAACT CCCTCAGAGT GAGACAGTGG TCCGAGGTCA

1921 GCAGTGGCGC TATCTTGGCT GACTGCAACC TCCGCCTCCC CGGTTCAAGC GATTCTCCTG
     CGTCACCGCG ATAGAACCGA CTGACGTTGG AGGCGGAGGG GCCAAGTTCG CTAAGAGGAC

1981 CCTCAGCCTC CTGAGTAGCT GGGACTACAG GAGCCCGCCA CCACGCCCAG CTAATTTTTG
     GGAGTCGGAG GACTCATCGA CCCTGATGTC CTCGGGCGGT GGTGCGGGTC GATTAAAAAC

2041 TATTTTTAGT AGAGATGGGG TTTCACCATG TTGGCCAGGA TGGTCTCGAT TTCTCGACTT
     ATAAAAATCA TCTCTACCCC AAAGTGGTAC AACCGGTCCT ACCAGAGCTA AAGAGCTGAA

2101 CGTGATCCGC CTGTCTGGGC CTCCCAAAGT GCTGGGATTA CAGGCGTGAG CCACCACGCC
     GCACTAGGCG GACAGACCCG GAGGGTTTCA CGACCCTAAT GTCCGCACTC GGTGGTGCGG

2161 CGGCTTTAAA AAATGGTTTT GTAATGTAAG TGGAGGATAA TACCCTACAT GTTTATTAAT
     GCCGAAATTT TTTACCAAAA CATTACATTC ACCTCCTATT ATGGGATGTA CAAATAATTA

2221 AACAATAATA TTCTTTAGGA AAAAGGGCGC GGTGGTGATT TACACTGATG ACAAGCATTC
     TTGTTATTAT AAGAAATCCT TTTTCCCGCG CCACCACTAA ATGTGACTAC TGTTCGTAAG

2281 CCGACTATGG AAAAAAAGCG CAGCTTTTTC TGCTCTGCTT TTATTCAGTA GAGTATTGTA
     GGCTGATACC TTTTTTTCGC GTCGAAAAAG ACGAGACGAA AATAAGTCAT CTCATAACAT

2341 GAGATTGTAT AGAATTTCAG AGTTGAATAA AAGTTCCTCA TAATTATAGG AGTGGAGAGA
     CTCTAACATA TCTTAAAGTC TCAACTTATT TTCAAGGAGT ATTAATATCC TCACCTCTCT
```

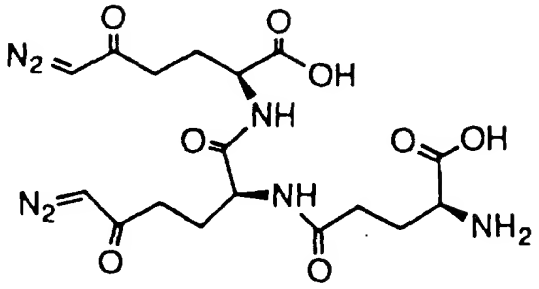
FIGURE 33



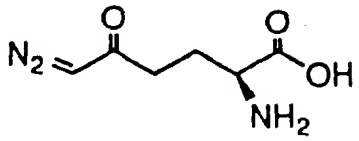
NAAG 1
N-acetylaspartyl-L-glutamate



Acivicin

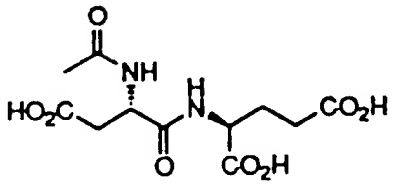


Azotomycin, becomes active by *in vivo* conversion to DON

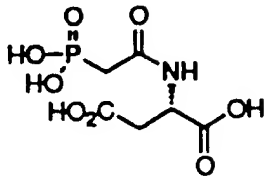


6-diazo-5-oxo-norleucine, DON

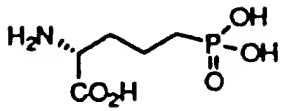
FIGURE 34



NAAG 1
N-acetylaspartyl-L-glutamate

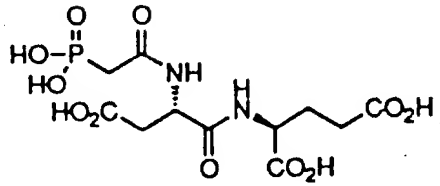


PALA 2
N-phosphonoacetyl-L-aspartate



D-APV

L-Glu antagonist



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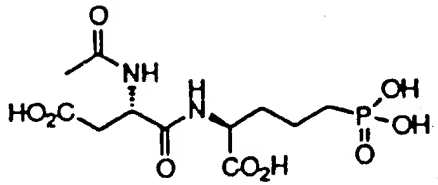

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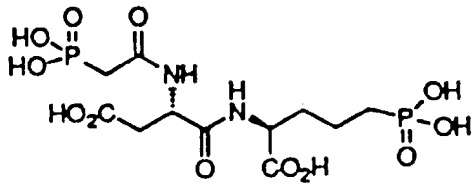
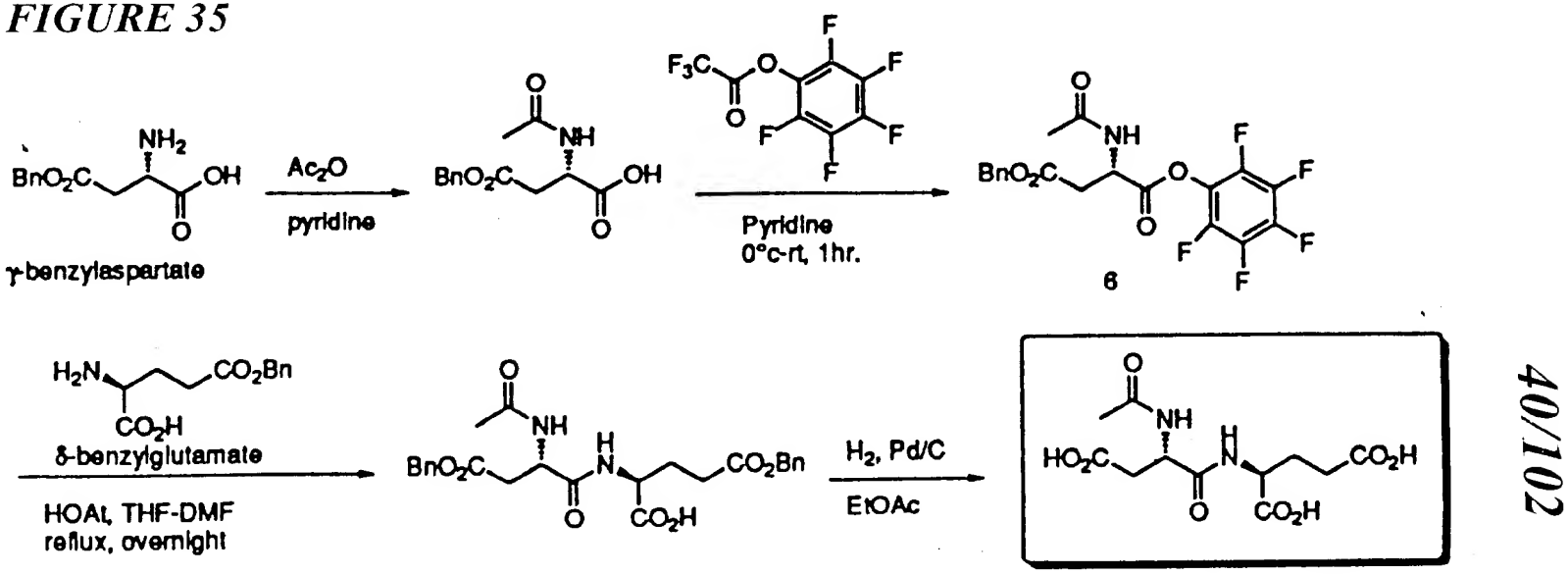


5

FIGURE 35



Ac_2O = acetic anhydride
THF = tetrahydrofurane
DMF = N,N-dimethylformamide
Pd/C = palladium on charcoal
EtOAc = ethylacetate

HOAt =

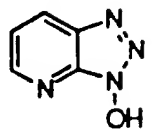


FIGURE 36

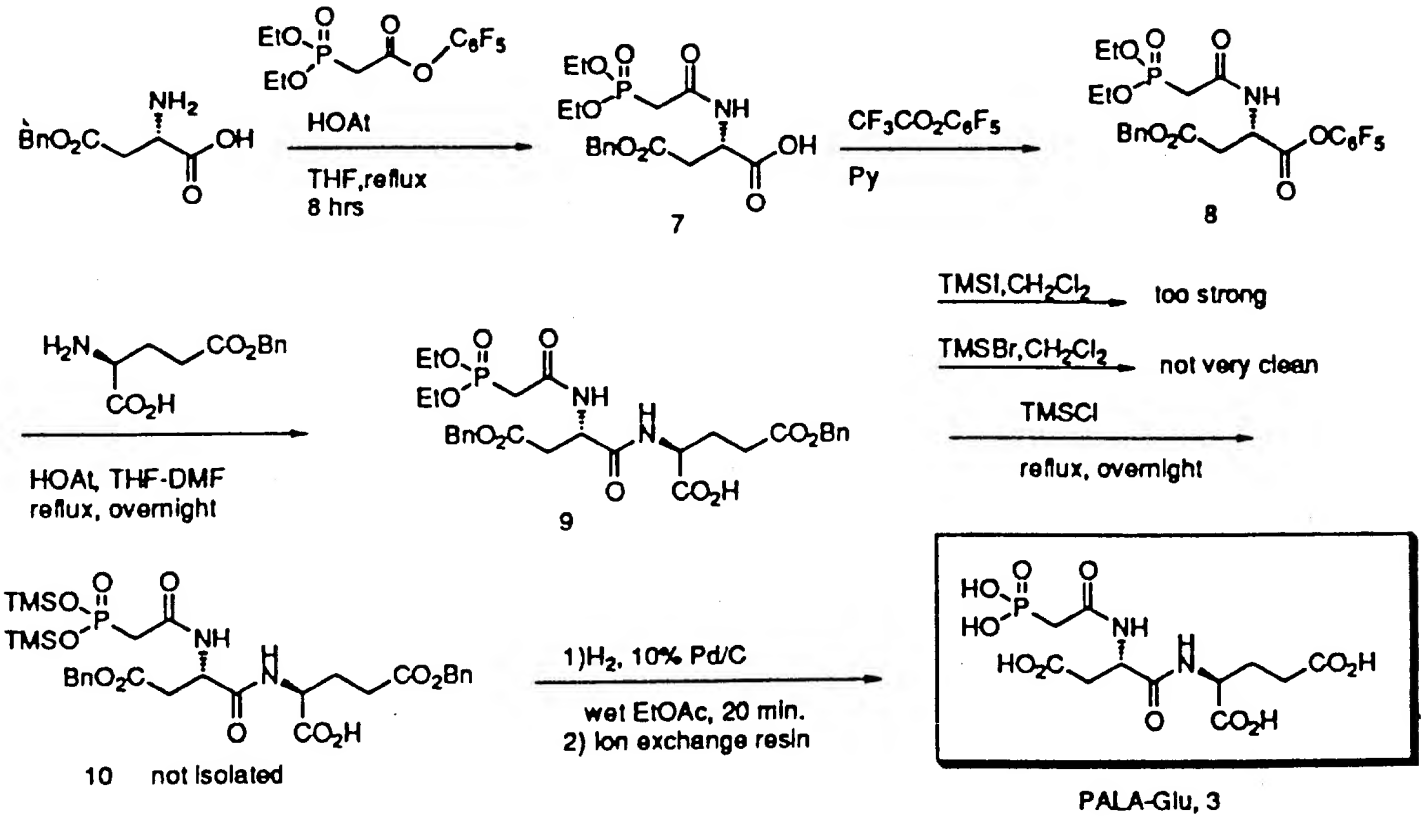

HOAt
THF,reflux
8 hrs
7
$CF_3CO_2C_6F_5$
Py
8
$TMSI,CH_2Cl_2$
too strong
$TMSBr,CH_2Cl_2$
not very clean
TMSCl
reflux, overnight
HOAt, THF-DMF
reflux, overnight
9
10 not isolated
1)H_2, 10% Pd/C
wet EtOAc, 20 min.
2) Ion exchange resin
PALA-Glu, 3

FIGURE 37

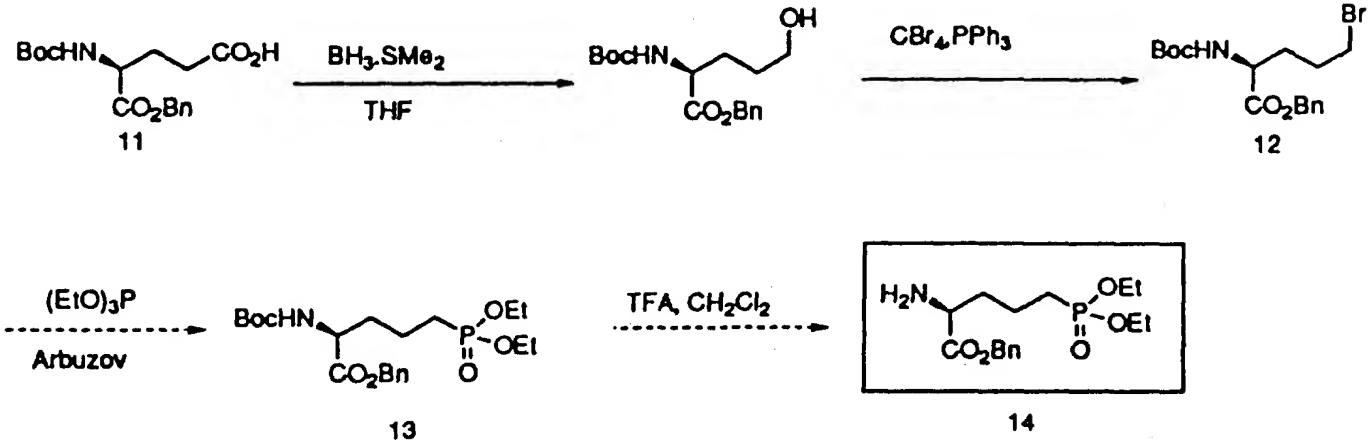

BocHN
CO2H
CO2Bn
11
BH3.SMe2
THF
OH
12
CBr4,PPh3
Br
(EtO)3P
Arbuzov
13
TFA, CH2Cl2
H2N
OEt
OEt
O
14

FIGURE 38

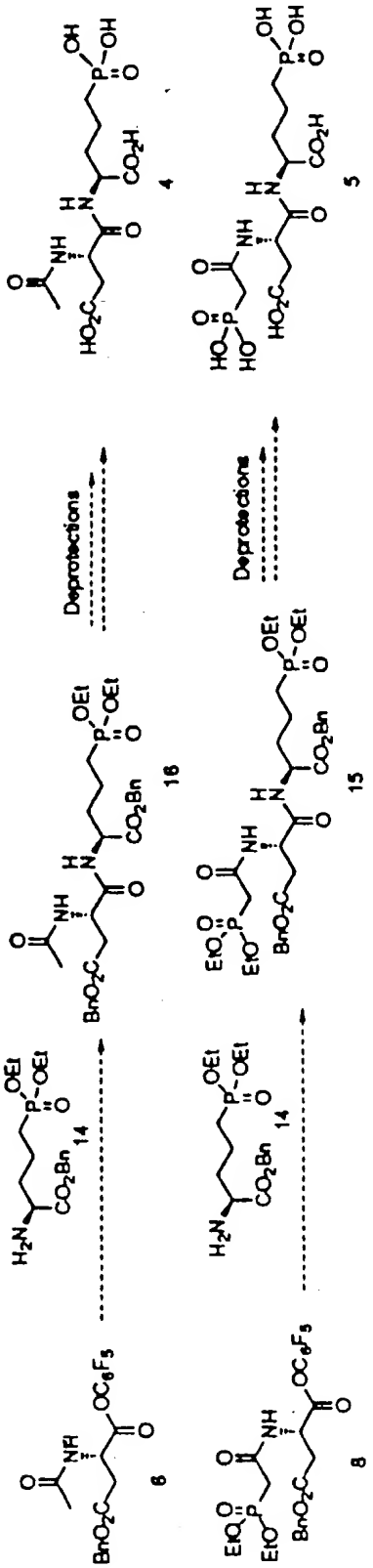
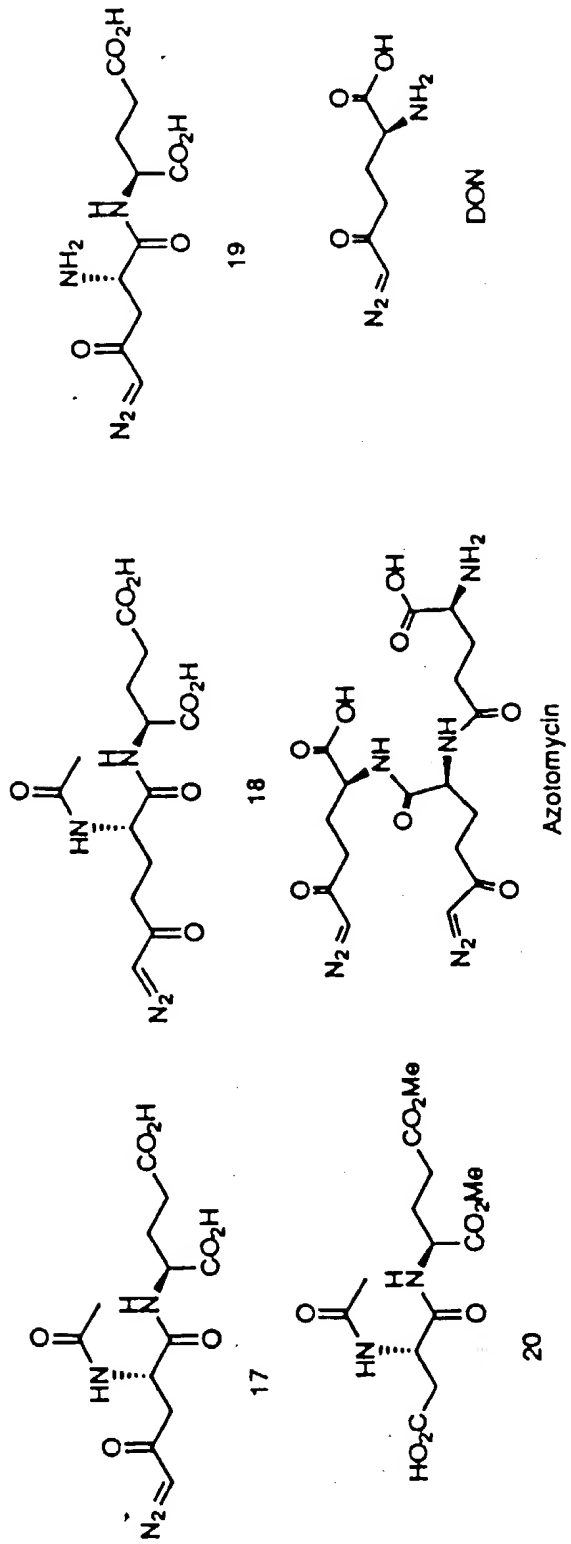
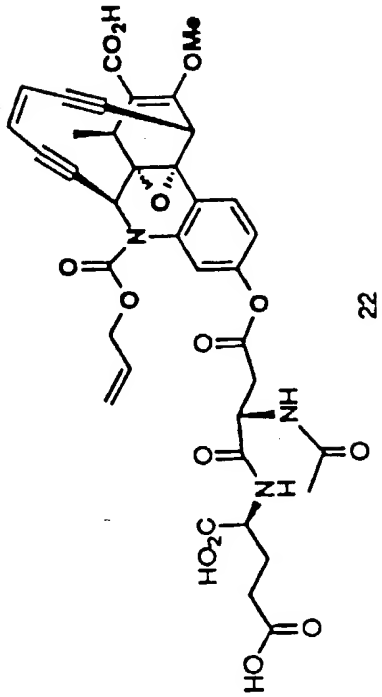


FIGURE 39





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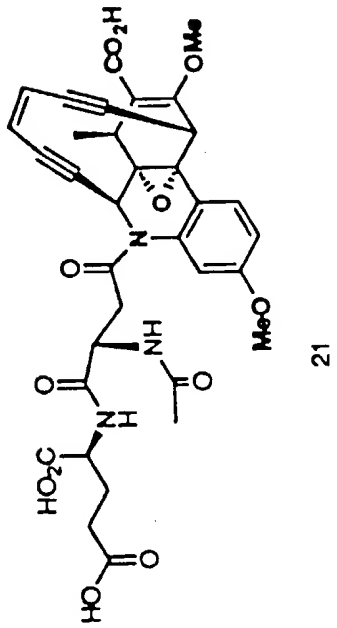


21

FIGURE 40

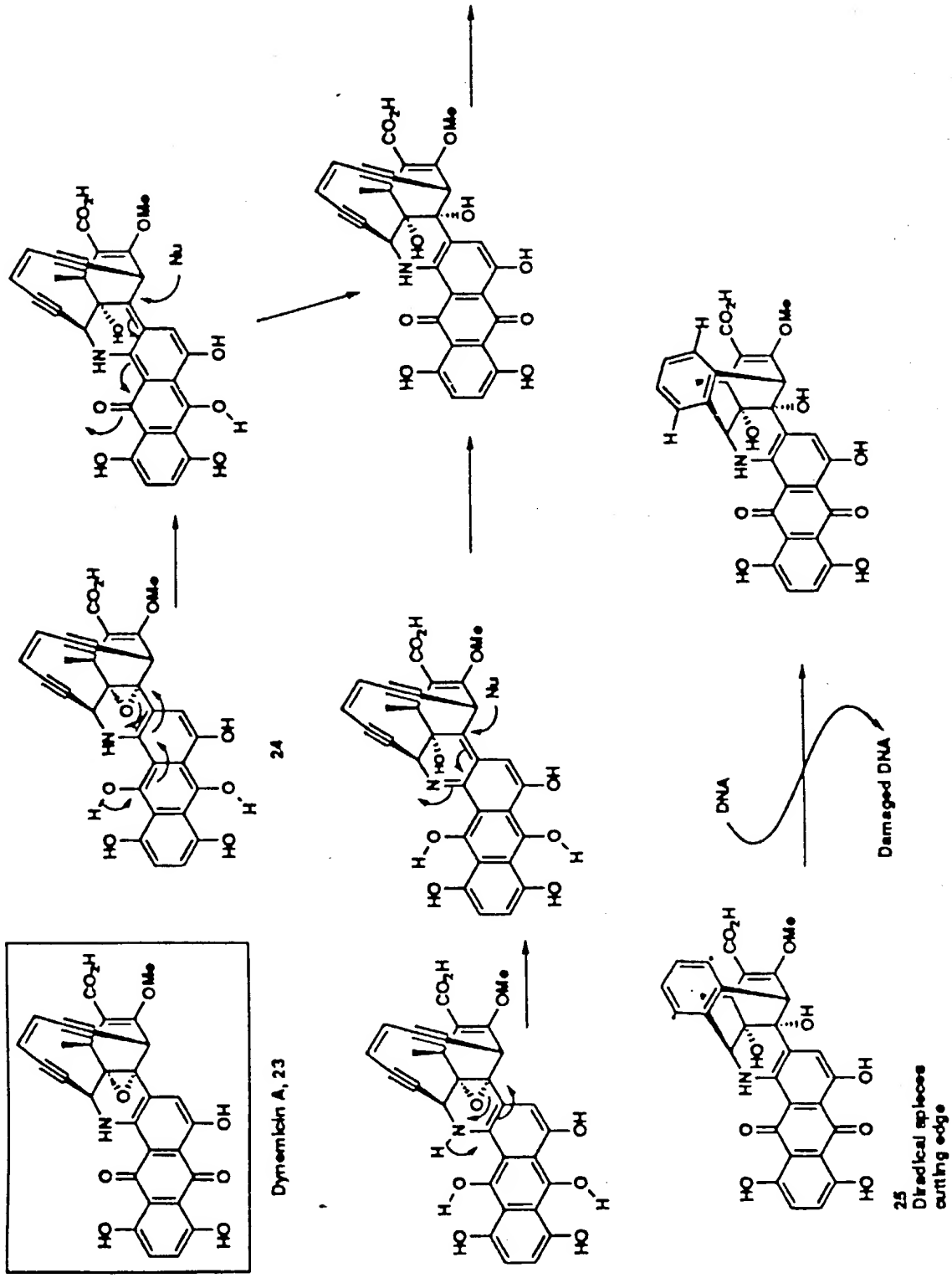
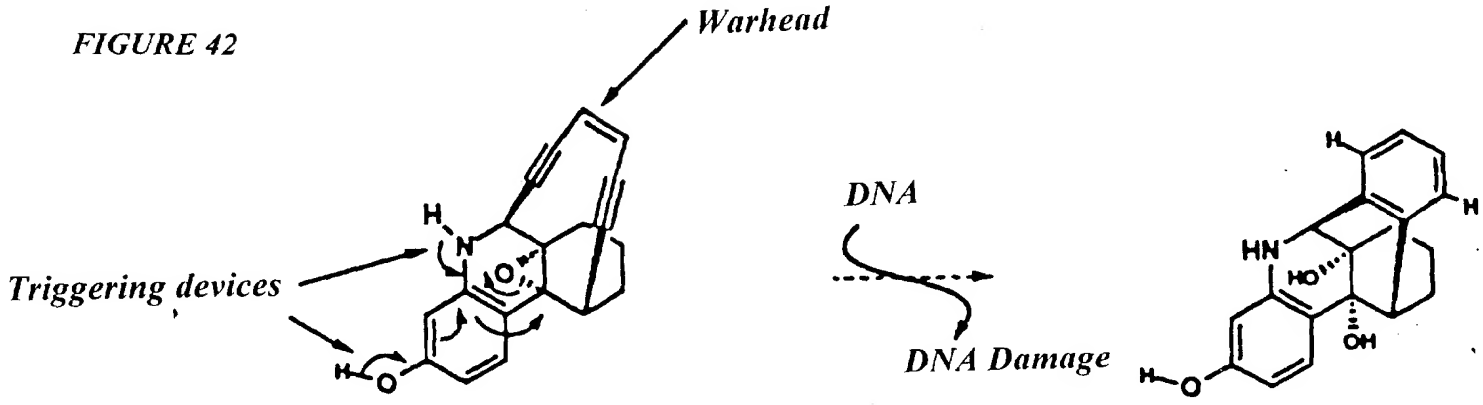

Dynemicin A, 23
24
DNA
Damaged DNA
25
Diradical spheres
cutting edge

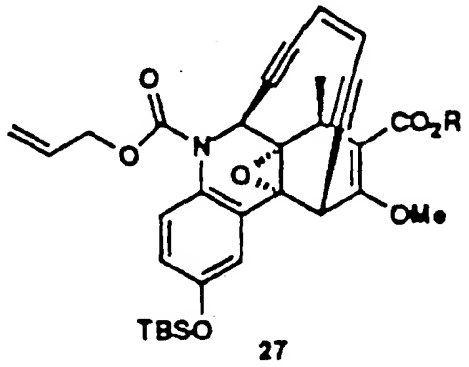
FIGURE 41

FIGURE 42



26

active at the nano to picomolar levels in different cell lines readily rearranges when one or both triggering devices are deprotected



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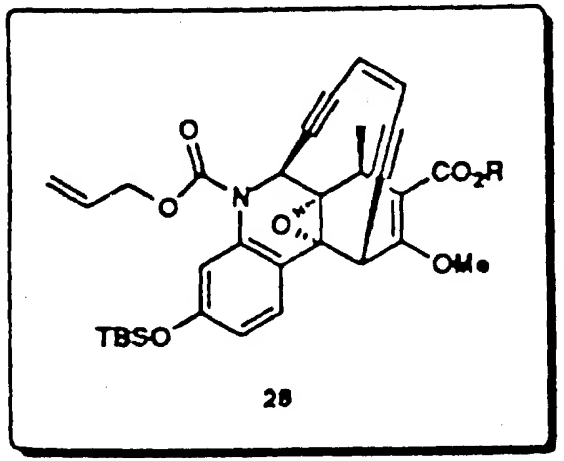

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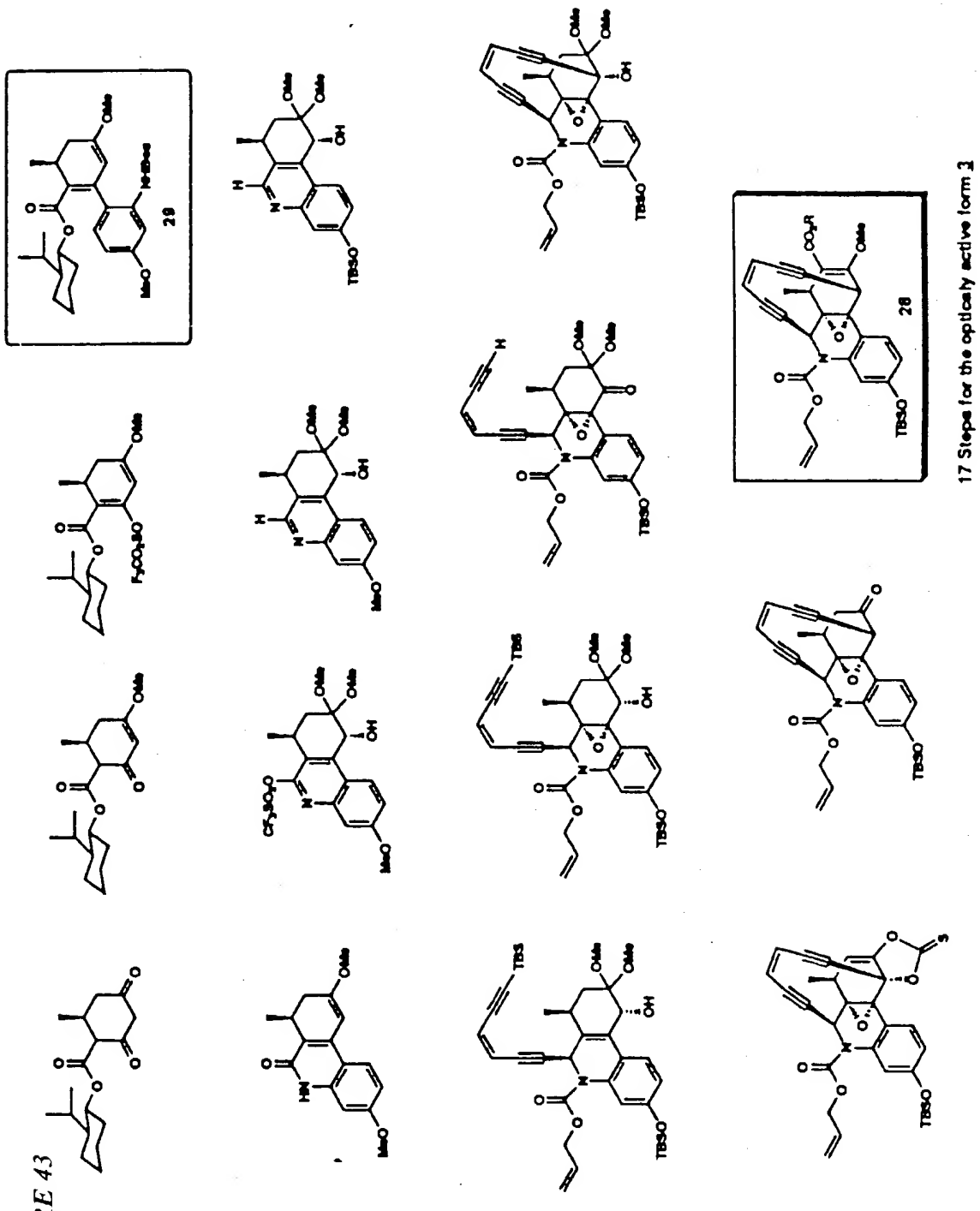


FIGURE 43

FIGURE 44

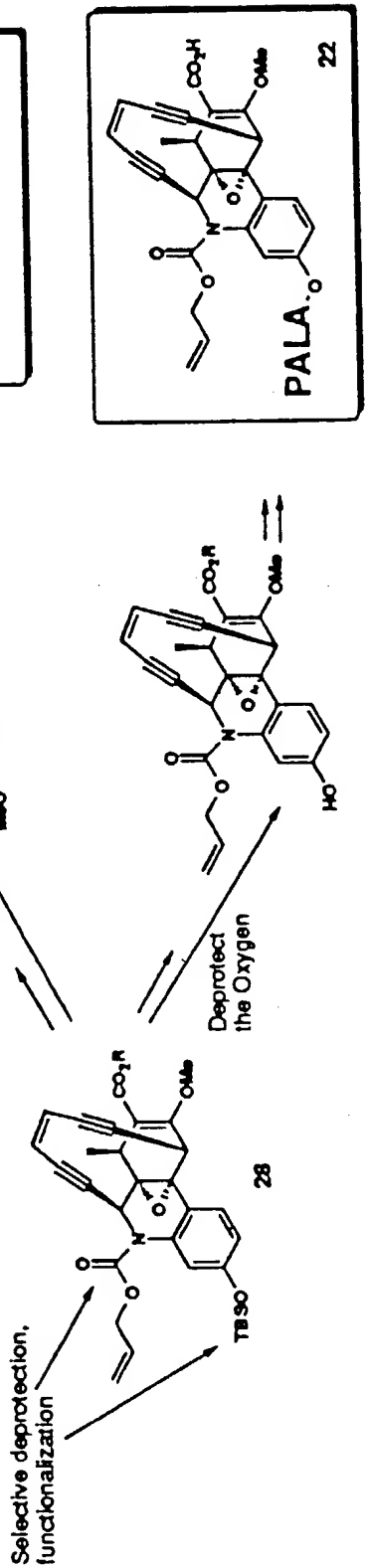

PALA
21
PALA
22
Deprotect the Nitrogen
Deprotect the Oxygen
Selective deprotection, functionalization
28

FIGURE 45

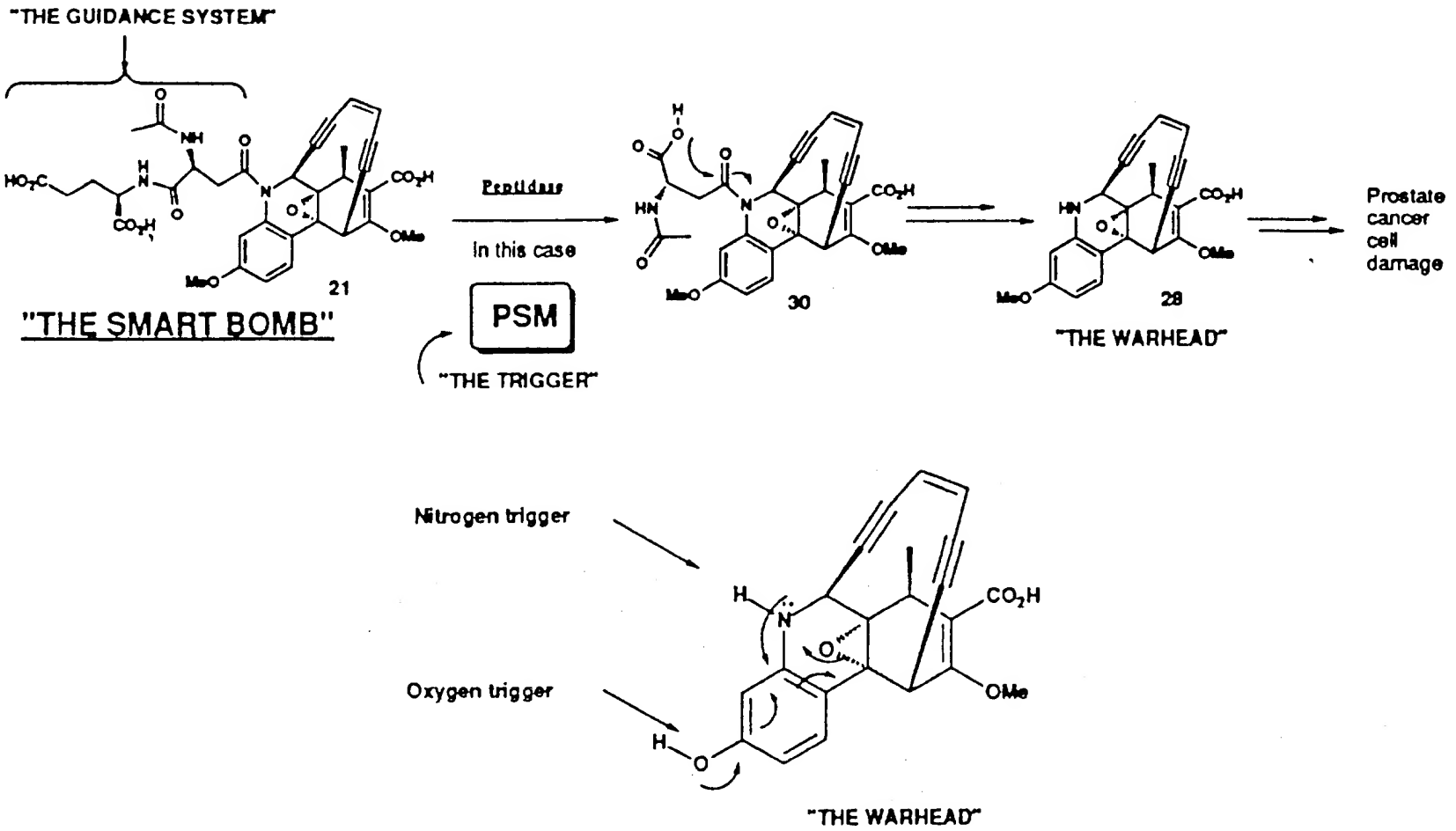

"THE GUIDANCE SYSTEM"
21
"THE SMART BOMB"
Peptidase
In this case
PSM
"THE TRIGGER"
30
28
"THE WARHEAD"
Prostate cancer cell damage
Nitrogen trigger
Oxygen trigger
"THE WARHEAD"

FIGURE 46A

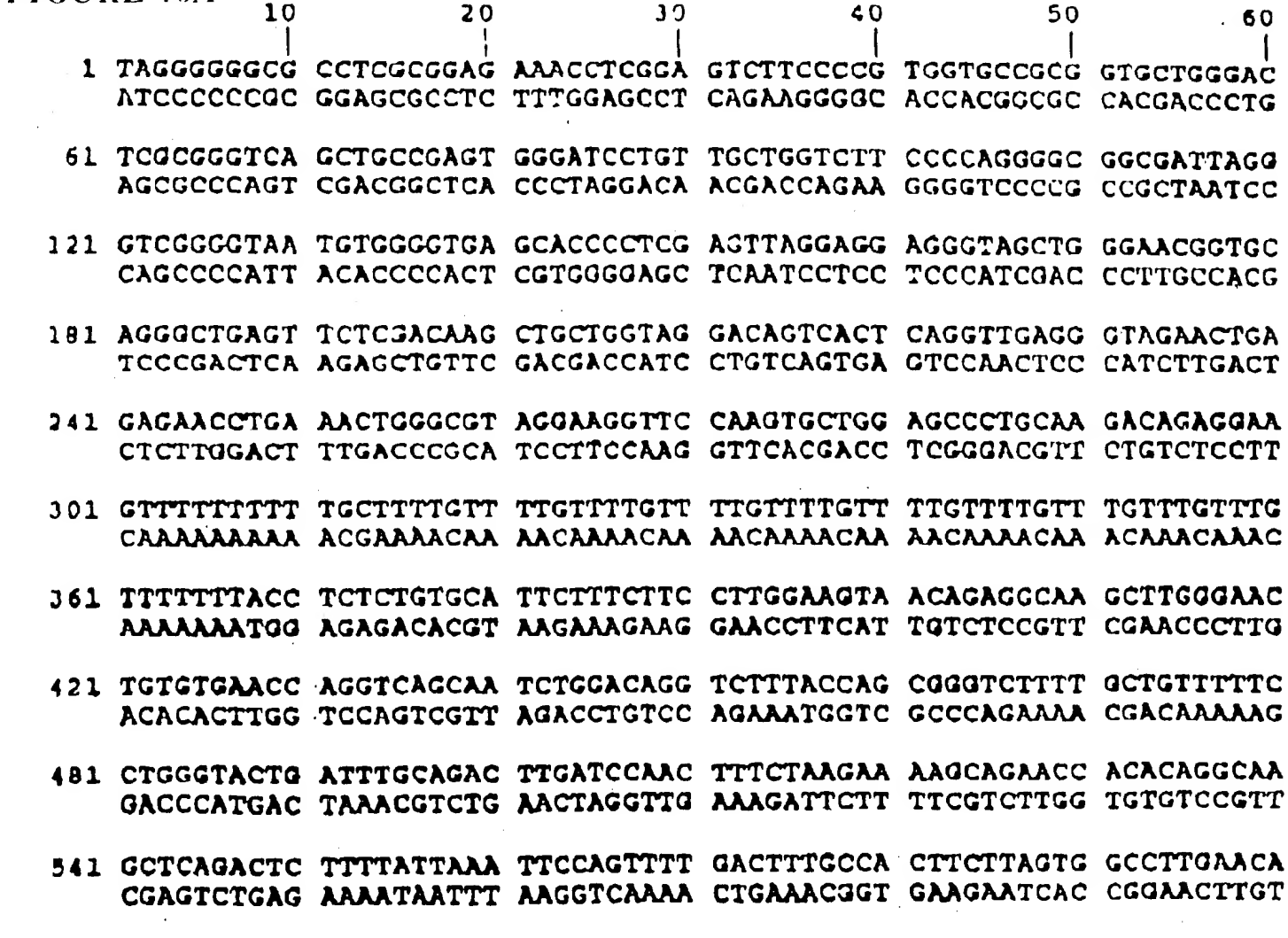

10 20 30 40 50 60
1 TAGGGGGGCG CCTCGCGGAG AAACCTCGGA GTCTTCCCCG TGGTGCCGCG GTGCTGGGAC
ATCCCCCCGC GGAGCGCCTC TTTGGAGCCT CAGAAGGGGC ACCACGGCGC CACGACCCTG
61 TCGCGGGTCA GCTGCCGAGT GGGATCCTGT TGCTGGTCTT CCCCAGGGGC GGCGATTAGG
AGCGCCCAGT CGACGGCTCA CCCTAGGACA ACGACCAGAA GGGGTCCCCG CCGCTAATCC
121 GTCGGGGTAA TGTGGGGTGA GCACCCCTCG AGTTAGGAGG AGGGTAGCTG GGAACGGTGC
CAGCCCCATT ACACCCCACT CGTGGGGAGC TCAATCCTCC TCCCATCGAC CCTTGCCACG
181 AGGGCTGAGT TCTCGACAAG CTGCTGGTAG GACAGTCACT CAGGTTGAGG GTAGAACTGA
TCCCGACTCA AGAGCTGTTC GACGACCATC CTGTCAGTGA GTCCAACTCC CATCTTGACT
241 GAGAACCTGA AACTGGGCGT AGGAAGGTTC CAAGTGCTGG AGCCCTGCAA GACAGAGGAA
CTCTTGGACT TTGACCCGCA TCCTTCCAAG GTTCACGACC TCGGGACGTT CTGTCTCCTT
301 GTTTTTTTTT TGCTTTTGTT TTGTTTTGTT TTGTTTTGTT TTGTTTTGTT TGTTTGTTTG
CAAAAAAAAA ACGAAAACAA AACAAAACAA AACAAAACAA AACAAAACAA ACAAACAAAC
361 TTTTTTTACC TCTCTGTGCA TTCTTTCTTC CTTGGAAGTA ACAGAGGCAA GCTTGGGAAC
AAAAAAATGG AGAGACACGT AAGAAAGAAG GAACCTTCAT TGTCTCCGTT CGAACCCTTG
421 TGTGTGAACC AGGTCAGCAA TCTGGACAGG TCTTTACCAG CGGGTCTTTT GCTGTTTTTC
ACACACTTGG TCCAGTCGTT AGACCTGTCC AGAAATGGTC GCCCAGAAAA CGACAAAAAG
481 CTGGGTACTG ATTTGCAGAC TTGATCCAAC TTTCTAAGAA AAGCAGAACC ACACAGGCAA
GACCCATGAC TAAACGTCTG AACTAGGTTG AAAGATTCTT TTCGTCTTGG TGTGTCCGTT
541 GCTCAGACTC TTTTATTAAA TTCCAGTTTT GACTTTGCCA CTTCTTAGTG GCCTTGAACA
CGAGTCTGAG AAAATAATTT AAGGTCAAAA CTGAAACGGT GAAGAATCAC CGGAACTTGT

FIGURE 46B

```
501 AGTTACCGAC TCCCTCTCAG CGTTAGTTAC CCTATTTTAT GATGAGGATA ATATTATCTG
    TCAATGGCTC AGGGAGAGTC GCAATCAATG GGATAAAATA CTACTCCTAT TATAATAGAC

661 CAAATTATTG GTAATAGTAA ATAATATAGC ATGTAAATCT CCTAGCACAG TACTGGGATT
    GTTTAATAAC CATTATCATT TATTATATCG TACATTTAGA GGATCGTGTC ATGACCCTAA

721 TTCGCCACTT TATTTCTTCT TTTACCAAGA TACTCCTCAT TGGACTTTAA TACACAGGAC
    AAGCGGTGAA ATAAAGAAGA AAATGGTTCT ATGAGGAGTA ACCTGAAATT ATGTGTCCTG

781 TAGTCTAAGG TATCACCAGG TAGTCCACTC CTGCTCGGAA TTCTTGACCC TCTTTCGGGA
    ATCAGATTCC ATAGTGGTCC ATCAGGTGAG GACGAGCCTT AAGAACTGGG AGAAAGCCCT

841 TTTAGAAGAA TAGGGCATGG ACCAGATGGG TTTAAACAAA TTCAATATCT TCCACTAGCT
    AAATCTTCTT ATCCCGTACC TGGTCTACCC AAATTTGTTT AAGTTATAGA AGGTGATCGA

901 TCACCTTGGG GTTGTTAAAA GATTTTTGAA CCACACACTG TGCTCATAAC AATCTTCATC
    AGTGGAACCC CAACAATTTT CTAAAAACTT GGTGTGTGAC ACGAGTATTG TTAGAAGTAG

961 TCTTAAAAGG ATTTTATTCT TCCTGGTATT GCCCTCACTC TCATCCCTGT ATTCCGTGCT
    AGAATTTTCC TAAAATAAGA AGGACCATAA CGGGAGTGAG AGTAGGGACA TAAGGCACGA
```

FIGURE 46C

```
1021 CAGTGGCTGA CACAGAAGAG TTCTTTATTG ATGTCCGCCC CCCACCCACT AGGATTCTCT
     GTCACCGACT GTGTCTTCTC AAGAAATAAC TACAGGCGGG GGGTGGGTGA TCCTAAGAGA

1081 GCTCTCCCCT CCCCCTACAG GCCTCCATCC TCTTCATCCT GTTCATTTTT CAGATCTCAG
     CGAGAGGGGA GGGGGATGTC CGGAGGTAGG AGAAGTAGGA CAAGTAAAAA GTCTAGAGTC

1141 TTCAAGCATC TCGTCCTCAG TGTGGTGTTT CCTGATCCCT CACTCTAATC CAAGTCTTTC
     AAGTTCGTAG AGCAGGAGTC ACACCACAAA GGACTAGGGA GTGAGATTAG GTTCAGAAAG

1201 TGTTTTATGC ACAGGTGGAA TCTTATTTCC GTTTGCGTCC AATCATGTAT TTTAATATGC
     ACAAAATACG TGTCCACCTT AGAATAAAGG CAAACGCAGG TTAGTACATA AAATTATACG

1261 ATGTATATAT GTATGTGCAT TTGTATGCAT GCGATTAAGA ACTAGAATAA TTAATAATTG
     TACATATATA CATACACGTA AACATACGTA CGCTAATTCT TGATCTTATT AATTATTAAC

1321 GAAAGCTCCA TGAAAGCTGG TTGGGGACTA ATTTTGTAAC TACTTTATTC CCAGATCCTG
     CTTTCGAGGT ACTTTCGACC AACCCCTGAT TAAAACATTG ATGAAATAAG GGTCTAGGAC

1381 TAATTTCTCT AAATAAACCC TGGAATCTTG CCTTATCTCC TTCAGGTTAA AAGCCAACTG
     ATTAAAGAGA TTTATTTGGG ACCTTAGAAC GGAATAGAGG AAGTCCAATT TTCGGTTGAC

1441 CAAGGTCTAA TGACTGCAGG ATCTAGCTAT CCATTGTTTC TGGCCGCCTA TGCGTGCACT
     GTTCCAGATT ACTGACGTCC TAGATCGATA GGTAACAAAG ACCGGCGGAT ACGCACGTGA

1501 GGGTGTCTGG CAGAGAGGCT GGGTAAATTG TAGTTTCATT GTAGCTGTCT GACTTGGATT
     CCCACAGACC GTCTCTCCGA CCCATTTAAC ATCAAAGTAA CATCGACAGA CTGAACCTAA

1561 TCTCACGCCT ACTTCACTGG AAACGCAAAC TCTCACAGCA TTTTGTTTTA GTTTCAGAAT
     AGAGTGCGGA TGAAGTGACC TTTGCGTTTG AGAGTGTCGT AAAACAAAAT CAAAGTCTTA

1621 CAGAGCAAAT TAGAAGTCTG AATTTCCTTC AACACTTGGA AATAATTTAT TTATTTGAAA
     GTCTCGTTTA ATCTTCAGAC TTAAAGGAAG TTGTGAACCT TTATTAAATA AATAAACTTT

1681 TATATTCATA ATTAATTCGT TATAAAAATG TATTAAATGC TTATTTGAGT CAGCAGAGGA
     ATATAAGTAT TAATTAAGCA ATATTTTTAC ATAATTTACG AATAAACTCA GTCGTCTCCT
```

FIGURE 46D

```
1741 AGATAGAAAC TTTATGAAAG TAGAAGGTGG ATCTCCTTTT TGCCTTCATT TTCAGAACAT
     TCTATCTTTG AAATACTTTC ATCTTCCACC TAGAGGAAAA ACGGAAGTAA AAGTCTTGTA

1801 CTCGTTTACA CCCATTAGTT GAAACATTAA TGTCATTTTA TTTTCGTCCT GATTATCTCA
     GAGCAAATGT GGGTAATCAA CTTTGTAATT ACAGTAAAAT AAAAGCAGGA CTAATAGAGT

1861 TAAAACATTT CTTAGAATAA CAGCAATACC TATCATTGAA GTTGGATAAG AAATATTTTG
     ATTTTGTAAA GAATCTTATT GTCGTTATGG ATAGTAACTT CAACCTATTC TTTATAAAAC

1921 CAATTGGTTT GCAACTTAAA AATCTGTTTG CATGACTCTT TTTCAGTGAA AGTAGGCAAG
     GTTAACCAAA CGTTGAATTT TTAGACAAAC GTACTGAGAA AAAGTCACTT TCATCCGTTC

1981 AGAAATTAAA ATTCAGAAAT ATCTCACCTA ATGTCAGAGG TAATATTGAT AATTTGTGTT
     TCTTTAATTT TAAGTCTTTA TAGAGTGGAT TACAGTCTCC ATTATAACTA TTAAACACAA

2041 TTACAAATAA TACATACAAC AATAATGAAA AATAAGTCCT ATCTATAGGC TCGTATCTCA
     AATGTTTATT ATGTATGTTG TTATTACTTT TTATTCAGGA TAGATATCCG AGCATAGAGT

2101 TGCCTATTTT TGGATGTATT TTTCA
     ACGGATAAAA ACCTACATAA AAAGT
```

FIGURE 47A


10 20 30 40 50 60
1 TGAAAAATAC ATCAAAAATA GGCATGAGAT ACGAGCCTAT AGATAGGACT TATTTTTTAT
ACTTTTTATG TAGTTTTTAT CCGTACTCTA TGCTCGGATA TCTATCCTGA ATAAAAAATA
61 TATTGTTGTA TGTATTATTT GTAAAACACA AATTATCAAT ATTACCTCTG ACATTAGGTG
ATAACAACAT ACATAATAAA CATTTTGTGT TTAATAGTTA TAATGGAGAC TGTAATCCAC
121 AGATATTCTG AATTTTAATT TCTCTTGCCT ACTTTCACTG AAAAAGAGTC ATGCAAACAG
TCTATAAGAC TTAAAATTAA AGAGAACGGA TGAAAGTGAC TTTTTCTCAG TACGTTTGTC
181 ATTTTTAAGT TGCAAACCAA TTGCAAAATA TTTTTTTATC CAACTTCAAT GATAGGTATT
TAAAAATTCA ACGTTTGGTT AACGTTTTAT AAAAAAATAG GTTGAAGTTA CTATCCATAA
241 GCTGTTAATT CTAAGATATG CATTAATTGT TTCAACTAAT GGGTGTCAAA CGAGATGTTC
CGACAATTAA GATTCTATAC GTAATTAACA AAGTTGATTA CCCACAGTTT GCTCTACAAG
301 TGAAAATGAA GGCAAAAAGG AGATCCACCT TCTACTTTCA TAAAGTTTCT ATCTTCCTCT
ACTTTTACTT CCGTTTTTCC TCTAGGTGGA AGATGAAAGT ATTTCAAAGA TAGAAGGAGA
361 GCTGACTCAA ATAAGCATTT AATACATTTT ATAACGAATT AATTATGAAT ATATTTCAAA
CGACTGAGTT TATTCGTAAA TTATGTAAAA TATTGCTTAA TTAATACTTA TATAAAGTTT
421 TAAATAAATT ATTTCCAAGT GTTGAAGGAA ATTCAGACTT CTAATTTGCT CTGATTCTGA
ATTTATTTAA TAAAGGTTCA CAACTTCCTT TAAGTCTGAA GATTAAACGA GACTAAGACT

FIGURE 47B

```
481 AACTAAAACA AATGCTCTGT GAGAGTTTGC GTTTCCAGTG AAGTAGCGTG AGAAATCCAA
    TTGATTTTGT TTACGAGACA CTCTCAAACG CAAAGGTCAC TTCATCGCAC TCTTTAGGTT

541 GTCAGACAGC TACATGAAAC TACATTTACC AGCTCTCTGC CAGACACCAG TGCACGATAG
    CAGTCTGTCG ATGTACTTTG ATGTAAATGG TCGAGAGACG GTCTGTGGTC ACGTGCTATC

601 CGCAGAACAT GTAGCTAGAT CTCAGTCATA GCTNNNNNNN NNNNNNNNNN AGACCTTGCA
    GCGTCTTGTA CATCGATCTA GAGTCAGTAT CGANNNNNNN NNNNNNNNNN TCTGGAACGT

661 GTTGGCTTTT AACCTGAAGG AGATAAGGCA AGATTCCAGG GTTTATTTAG AGAAATTACA
    CAACCGAAAA TTGGACTTCC TCTATTCCGT TCTAAGGTCC CAAATAAATC TCTTTAATGT

721 GGATCTGGGA ATAAAGTAGT TACAAAATTA GTCCCCAACC AGCTTTCATG GAGCTTTCAA
    CCTAGACCCT TATTTCATCA ATGTTTTAAT CAGGGGTTGG TCGAAAGTAC CTCGAAAGTT
```

FIGURE 47C

```
781 TTATTAATTA TTCTAGTTCT TAATCGCATG CATACAATGC ACATACATAT ATACATGCAT
    AATAATTAAT AAGATCAAGA ATTAGCGTAC GTATGTTACG TGTATGTATA TATGTACGTA

841 ATTAAAATAC ATGATTGGAC GCAAACGGAA ATAAGATTCC ACCTGTGCAT AAAACAGAAA
    TAATTTTATG TACTAACCTG CGTTTGCCTT TATTCTAAGG TGGACACGTA TTTTGTCTTT

901 GACTTGGTTA GAGTGAGGGA TCAGGAAACA CCACACTGAG GACGAGATGN NNNNNNNNNN
    CTGAACCAAT CTCACTCCCT AGTCCTTTGT GGTGTGACTC CTGCTCTACN NNNNNNNNNN

961 NTAGTGGGTG GGGGGCGGAC ATCAATAAAG AACTCTTCTG TGTCAGCCAC TGAGCACGGA
    NATCACCCAC CCCCCGCCTG TAGTTATTTC TTGAGAAGAC ACAGTCGGTG ACTCGTGCCT

1021 ATAAAGGGAT GAGAGTGAGG GCAANTACCA GAAGAATAAA ATCCTTTTAA GAGATGAAGA
     TATTTCCCTA CTCTCACTCC CGTTNATGGT CTTCTTATTT TAGGAAAATT CTCTACTTCT

1081 TTGTTATGAG CACAGTGTGT GGNTTCAAAA ATCTTTTAAC AACCCCAAGG TGAAGCTAGT
     AACAATACTC GTGTCACACA CCNAAGTTTT TAGAAAATTG TTGGGGTTCC ACTTCGATCA

1141 TGGAAGATAT TTGAATTTGT TTAAACCCAT CTGGTCCTAG CCCTATTCTT TGAATCCCGA
     ACCTTCTATA AACTTAAACA AATTTGGGTA GACCAGGATC GGGATAAGAA ACTTAGGGCT
```

FIGURE 47D

```
1201 AAGAGGGTCA AGAATTCCGA GCAGGAGTGG ACTACCTGGT GATACCTTAG ACTAGTCCTG
     TTCTCCCAGT TCTTAAGGCT CGTCCTCACC TGATGGACCA CTATGGAATC TGATCAGGAC

1261 TGTATTAAAG TCCAATGAGG AGTATCTTGG TAAAATAATA AATAAAGTCC CGAAAATCCC
     ACATAATTTC AGGTTACTCC TCATAGAACC ATTTTATTAT TTATTTCAGG GCTTTTAGGG

1321 AGTACTGTGC TAGGAGATTT ACATGCTATA TTATTTACTA TNNNNNNNNT AATTTGCAGA
     TCATGACACG ATCCTCTAAA TGTACGATAT AATAAATGAT ANNNNNNNNA TTAAACGTCT

1381 TAATATTATC CTCATCATAA AATAGGGTAA CTAACGCTGA GAGGGACTCG GTAACTTGTT
     ATTATAATAG GAGTAGTATT TTATCCCATT GATTGCGACT CTCCCTGAGC CATTGAACAA

1441 CAAGGCCACT AAGAAGTGGC AAAGTCAAAA CTGGAATTTT AATAAAAGAG TCTAGCTTGC
     GTTCCGGTGA TTCTTCACCG TTTCAGTTTT GACCTTAAAA TTATTTTCTC AGATCGAACG

1501 CTGTGTGGTT CTGCTTTTCT TAGAAAGTTG GANNAAGTCT CANATCAGTA CCCAGGAAAA
     GACACACCAA GACGAAAAGA ATCTTTCAAC CTNNTTCAGA GTNTAGTCAT GGGTCCTTTT

1561 ACAGCAAAAG ACCCGCTGGT AAAGACCTGT CCAGATTGCT GACCTGGTTC ACACANNTCC
```

FIGURE 47E

```
     TGTCGTTTTC TGGGCGACCA TTTCTGGACA GGTCTAACGA CTGGACCAAG TGTGTNNAGG

1621 AAGCTTGCCT CTGTTACTTC CAAGGAACAA AGAATGCACA GAGAGGTAAA AAAACAAACA
     TTCGAACGGA GACAATGAAG GTTCCTTCTT TCTTACGTGT CTCTCCATTT TTTTGTTTGT

1681 AACCAAACAA AACAAAACAA AACAAAACAA AACAAAACAA AAGCAAAAAA AAACTTCCTC
     TTGGTTTGTT TTGTTTTGTT TTGTTTTGTT TTGTTTTGTT TTCGTTTTTT TTTGAAGGAG

1741 TGTCTTGCAG GGCTCCAGCA CTTGGAACCT TCCTACGTCC TANTTTCAGG TTCTCTCAGT
     ACAGAACGTC CCGAGGTCGT GAACCTTGGA AGGATGCAGG ATNAAAGTCC AAGAGAGTCA

1801 TCTACCCTCA ACCTGAGTGA CTGTCCTACC AGCAGCTTGT CGAGAACTCA GCCCTGCACC
     AGATGGGAGT TGGACTCACT GACAGGATGG TCGTCGAACA GCTCTTGAGT CGGGACGTGG

1861 GTTCCCAGCT ACCCTCCTCC TAACTCGAGG GGTGCT
     CAAGGGTCGA TGGGAGGAGG ATTGAGCTCC CCACGA
```

FIGURE 48A

```
                10          20          30          40          50          60
                |           |           |           |           |           |
  1 GGATTCTGTT GAGCCCTAGC TCATTATGAT GTCCTGTTGT CCTACCCAAA TAAGACTCAT
    CCTAAGACAA CTCGGGATCG AGTAATACTA CAGGACAACA GGATGGGTTT ATTCTGAGTA

 61 CCCAACTACA TCTCAATAAT TAATGAAGAT GGAAATGAGG TAAAAAATAA ATAAATAAAT
    GGGTTGATGT AGAGTTATTA ATTACTTCTA CCTTTACTCC ATTTTTTATT TATTTATTTA

121 AAAAGAAACA TTCCCCCCCA TTTATTATTT TTTCAAATAC CTTCTATGAA ATAATGTTCT
    TTTTCTTTGT AAGGGGGGGT AAATAATAAA AAAGTTTATG GAAGATACTT TATTACAAGA

181 ATCCCTCTCT AAATATTAAT AGAAATCAAT ATTATTGGAA CTGTGAATAC CTTTAATATC
    TAGGGAGAGA TTTATAATTA TCTTTAGTTA TAATAACCTT GACACTTATG GAAATTATAG

241 TCATTATCCG GTGTCAACTA CTTTCCTATG ATGTTGAGTT ACTGGGTTTA GAAGTCGGGA
    AGTAATAGGC CACAGTTGAT GAAAGCATAC TACAACTCAA TGACCCAAAT CTTCAGCCCT

301 AATAATGCTG TAAANNNNNN AGTTAGTCTA CACACCAATA TCAAATATGA TATACTTGTA
    TTATTACGAC ATTTNNNNNN TCAATCAGAT GTGTGGTTAT AGTTTATACT ATATGAACAT

361 AACCTCCAAG CATAAAAAGA GATACTTTAT AAAAGAGGTT CTTTTTTTCT TTTTTTTTTT
    TTGGAGGTTC GTATTTTTCT CTATGAAATA TTTTCTCCAA GAAAAAAAGA AAAAAAAAAA
```

FIGURE 48B

```
421 TCCAGATGGA GTTTCACTCC TGTCAGGCAG GCNGAGTGCA GTGGTGCCAT CTCGGCTCAC
    AGGTCTACCT CAAAGTGAGG ACAGTCCGTC CGNCTCACGT CACCACGGTA GAGCCGAGTG

481 TGCAACCTCC ACCTCCCATG TTCAAGGGAT TCTCCTTCCT CAGTCTCCTG AGTAGCTGGG
    ACGTTGGAGG TGGAGGGTAC AAGTTCCCTA AGAGGAAGGA GTCAGAGGAC TCATCGACCC

541 ATTACAGGTG TGCACCACCA CACCCAGCTA ATTTTTGTAT TTTTAATAGA GACAGGGTTT
    TAATGTCCAC ACGTGGTGGT GTGGGTCGAT TAAAAACATA AAAATTATCT CTGTCCCAAA

601 CATCGATGTT GGCCAGGCTA GTCTCGAACT CCTGACCTCT AGGTGATCCA CCCGCCTCAG
    GTAGCTACAA CCGGTCCGAT CAGAGCTTGA GGACTGGAGA TCCACTAGGT GGGCGGAGTC

661 CCTCCCAAAG TTGTAGAATT ACACGTGTGA GGCACTGCTC TGGCCAGGAG ATACATTTTT
    GGAGGGTTTC AACATCTTAA TGTGCACACT CCGTGACGAG ACCGGTCCTC TATGTAAAAA

721 GATAGGTTTA ATTTATAAAG ACACTGCACA GATTTGGAGT TGCTGGGAAA TCACGATCCA
    CTATCCAAAT TAAATATTTC TGTGACGTGT CTAAACCTCA ACGACCCTTT AGTGCTAGGT
```

FIGURE 48C

```
781 GTATGCATTT GACCCAGCAA TTTTTATTGG TACTTAATGA TTATATCTCA ATTGATCAGG
    CATACGTAAA CTGGGTCGTT AAAAATAACC ATGAATTACT AATATAGAGT TAACTAGTCC

841 TTGAACTCTG TGCGAAGAAT TTGTGTGTGG ACATTTGAGA GGACAGTTTG GAGGCAAGGT
    AACTTGAGAC ACGCTTCTTA AACACACACC TGTAAACTCT CCTGTCAAAC CTCCGTTCCA

901 ATTTTAGTAG ATTTAAAGAA TTTGAATCTT GTTTGCAAGT TGGGGCATAT ACTGAGAAAG
    TAAAATCATC TAAATTTCTT AAACTTAGAA CAAACGTTCA ACCCCCGTATA TGACTCTTTC

961 AGAAGACAAT GCAGATAAAT TGATATATTT ATTATGATGT ATGTTCAATA TGAAAGATCA
    TCTTCTGTTA CGTCTATTTA ACTATATAAA TAATACTACA TACAAGTTAT ACTTTCTAGT

1021 CAAAATATAA CATACATNNA TCTTACTTAA CATACCTCAG TTTTAGAGCT ACCGTATGTA
     GTTTTATATT GTATGTANNT AGAATGAATT GTATGGAGTC AAAATCTCGA TGGCATACAT

1081 GAAGAGTCCA TTTCTATTTA GGTAAGTTCC TTTAGTCCTT TTATTACTGG GCACTCTTAA
     CTTCTCAGGT AAAGATAAAT CCATTCAAGG AAATCAGGAA AATAATGACC CGTGAGAATT

1141 TTACATGTAG CTTGAAATAT GTCCAGTTTG AGCAGTGAAC TGAAAATGTC ATGTGATTAA
     AATGTACATC GAACTTTATA CAGGTCAAAC TCGTCACTTG ACTTTTACAG TACACTAATT

1201 GTACATATAT AATTTTTTTT CATAGTAGGT CAATAACCTC CTTTTATTGA CTAATGAATC
     CATGTATATA TTAAAAAAAA GTATCATCCA GTTATTGGAG GAAAATAACT GATTACTTAG

1261 AGTTCTCTAA TGATTATACG
     TCAAGAGATT ACTAATATGC
```

FIGURE 49A

```
                10        20        30        40        50        60
                |         |         |         |         |         |
  1 AATCAAAATA AAACAGTTAA AGTTTGATTA CTATAATCAA ACACAAAAAA AATGAATATT
    TTAGTTTTAT TTTGTCAATT TCAAACTAAT GATATTAGTT TGTGTTTTTT TTACTTATAA

 61 ATCTTTTATG TCAGTAGAGG GTGAATGAAT CCTTCAGGAT TTTGATGATA GTATCAGATA
    TAGAAAATAC AGTCATCTCC CACTTACTTA GGAAGTCCTA AAACTACTAT CATAGTCTAT

121 CCCAGCACTA TGCTAGAAGT TGTGAAGAAT TCACGAGATG AATAAATCAC AGATTCTGTC
    GGGTCGTGAT ACGATCTTCA ACACTTCTTA AGTGCTCTAC TTATTTAGTG TCTAAGACAG

181 CTCAAAATGG TTAGATCTAT TCAGGAAACA AAGCTAAAAA AACCCCACCA ATAACTAAAA
    GAGTTTTACC AATCTAGATA AGTCCTTTGT TTCGATTTTT TTGGGGTGGT TATTGATTTT

241 ATCAACCAAA TGAAAAACAA CAATCATAAA ATAAGTAAGT ACCTATAGAA AGAAAAGCTC
    TAGTTGGTTT ACTTTTTGTT GTTAGTATTT TATTCATTCA TGGATATCTT TCTTTTCGAG

301 AGAGGAGGTA AAAAGATAAC TCTTCCAAAA GGAATACTAT ATACTGTAAA CTGTGTACTG
    TCTCCTCCAT TTTTCTATTG AGAAGGTTTT CCTTATGATA TATGACATTT GACACATGAC

361 ATAGAAGGAA GAATTAGAAA NNNNNNNNTG TAAGTGGCAT ACATACTAAG CTAGTGTGAA
    TATCTTCCTT CTTAATCTTT NNNNNNNNAC ATTCACCGTA TGTATGATTC GATCACACTT
```

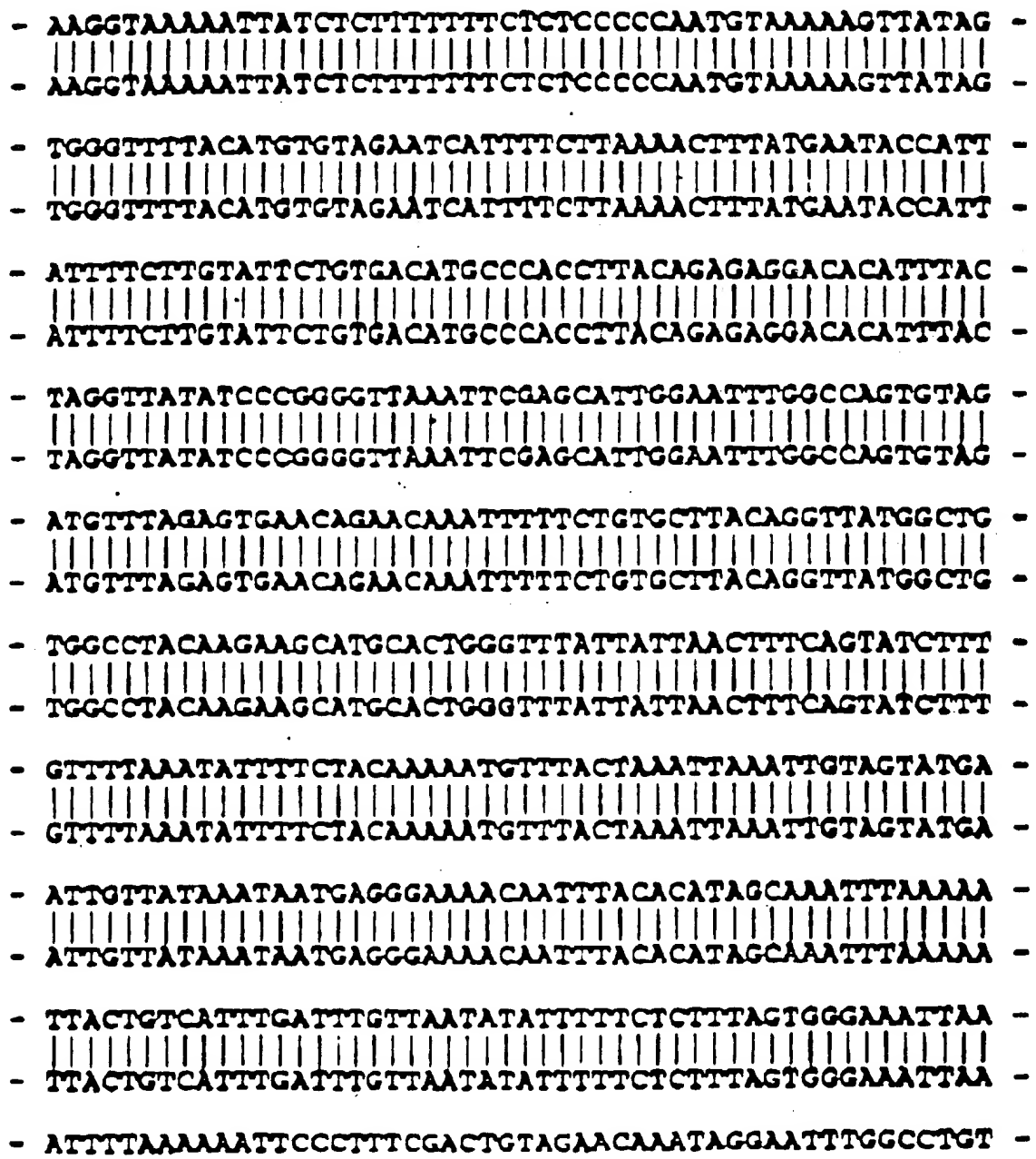
FIGURE 49B

```
421 CACAAGCCTA AATATGTAGT TGCTTCACAG AAGGTTAGAA GTAAATTAAC CTCATGAATT
    GTGTTCGGAT TTATACATCA ACGAAGTGTC TTCCAATCTT CATTTAATTG GAGTACTTAA

481 TCTTGAGAGA ACTTGTAAGG ACTAAGCTTT CGATTTTGGA GAAAGATTTT AATACCAAAT
    AGAACTCTCT TGAACATTCC TGATTCGAAA GCTAAAACCT CTTTCTAAAA TTATGGTTTA

541 AAAAAGTACC TTTGTTTGGT AATCTCAATC ATTATAATAG TGCTTAGATA ATACCTAGGA
    TTTTTCATGG AAACAAACCA TTAGAGTTAG TAATATTATC ACGAATCTAT TATGGATCCT

601 ACAAATTAAA TATTAAATTT ACTTTAAAAA AAAGTACATG ATTGGGGAAT CACAACTGGC
    TGTTTAATTT ATAATTTAAA TGAAATTTTT TTTCATGTAC TAACCCCTTA GTGTTGACCG

661 CTTACTAGAT TCTCTNNNNN NATATGCACT GAAAAGAATG AAAAACACTG AACCAAATAT
    GAATGATCTA AGAGANNNNN NTATACGTGA CTTTTCTTAC TTTTTGTGAC TTGGTTTATA

721 NTGTTTTTTT AAGTTTAAAA TTAAATTGGA AAAAAATAGT AAGGAATATC AGAAGCAAAA
    NACAAAAAAA TTCAAATTTT AATTTAACCT TTTTTTATCA TTCCTTATAG TCTTCGTTTT
```

FIGURE 49C

```
781 AAATAAAATG AAAGCAAGAA TCCTCAGAGG TAGCACGAAA TTTGGCTTTG CTTAGATGGA
    TTTATTTTAC TTTCGTTCTT AGGAGTCTCC ATCGTGCTTT AAACCGAAAC GAATCTACCT

841 TCTATCAAAG CTATGGCCCA TGAAAAGGAT TCAGGAGTTA GTTTAAAGCT GGTTCACATA
    AGATAGTTTC GATACCGGGT ACTTTTCCTA AGTCCTCAAT CAAATTTCGA CCAAGTGTAT

901 ATGGAATCTA GCAGAAGACT GTGCATAAAG GTGGTCTAAG AACAACAATA TCCTGACCAG
    TACCTTAGAT CGTCTTCTGA CACGTATTTC CACCAGATTC TTGTTGTTAT AGGACTGGTC

961 GTGAGGGGGC TCACNCTNAA TNCCAGCACT TTGGGAGCCC AAGGTGGGTG GATCACGAGG
    CACTCCCCCG AGTGNGANTT ANGGTCGTGA AACCCTCGGG TTCCACCCAC CTAGTGCTCC

1021 TCAGGAGTTT GAGACCAGCC TGACCAACAT GGTGAAACCG CGTCTCTACT AAAAATAGAA
     AGTCCTCAAA CTCTGGTCGG ACTGGTTGTA CCACTTTGGC GCAGAGATGA TTTTTATCTT

1081 AAATTAGCCG NGCCTACGTG CTTCTAATCC CAGCTGAACT CAGGAGACTG AGACAGGAGA
     TTTAATCGGC NCGGATGCAC GAAGATTAGG GTCGACTTGA GTCCTCTGAC TCTGTCCTCT

1141 ATCACTTGAA CCCAGCATGC AAGCTTNNNN NNGCCACTGC ACTCCAGCCT AGGGTGCAAA
     TAGTGAACTT GGGTCGTACG TTCGAANNNN NNCGGTGACG TGAGGTCGGA TCCCACGTTT

1201 AAAAAAAAAA ANGACACATT ACTCAGGTAA GGTAATCAAT AA
     TTTTTTTTTT TNCTGTGTAA TGAGTCCATT CCATTAGTTA TT
```

FIGURE 50A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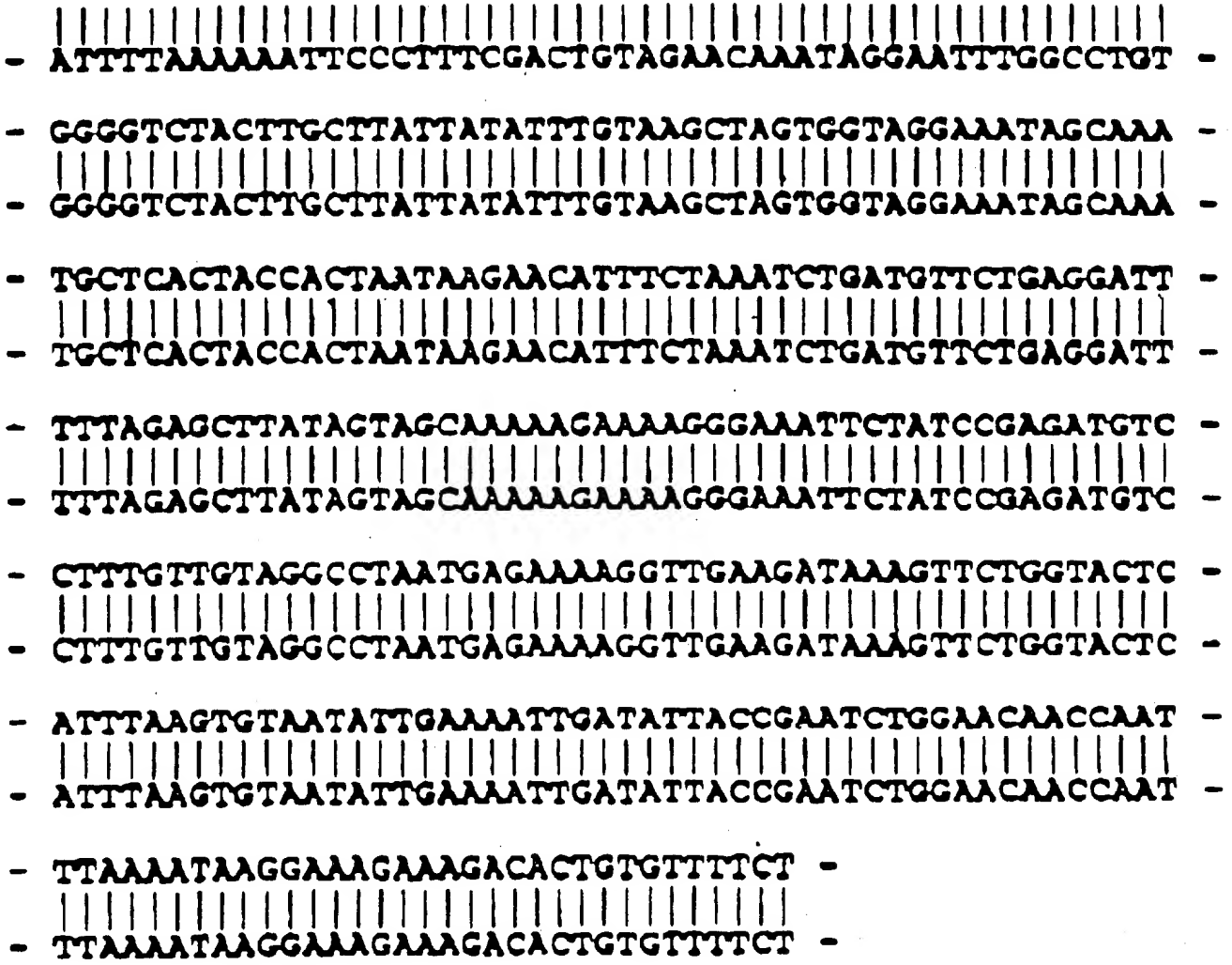
- TGGGTTTTACATGTGTAGAATCATTTTCTTAAAACTTTATGAATACCATT -
- ATTTTCTTGTATTCTGTGACATGCCCACCTTACAGAGAGGACACATTTAC -
- ATTTTCTTGTATTCTGTGACATGCCCACCTTACAGAGAGGACACATTTAC -
- TAGGTTATATCCCGGGGTTAAATTCGAGCATTGGAATTTGGCCAGTGTAG -
- TAGGTTATATCCCGGGGTTAAATTCGAGCATTGGAATTTGGCCAGTGTAG -
- ATGTTTAGAGTGAACAGAACAAATTTTTCTGTGCTTACAGGTTATGGCTG -
- ATGTTTAGAGTGAACAGAACAAATTTTTCTGTGCTTACAGGTTATGGCTG -
- TGGCCTACAAGAAGCATGCACTGGGTTTATTATTAACTTTCAGTATCTTT -
- TGGCCTACAAGAAGCATGCACTGGGTTTATTATTAACTTTCAGTATCTTT -
- GTTTTAAATATTTTCTACAAAAATGTTTACTAAATTAAATTGTAGTATGA -
- GTTTTAAATATTTTCTACAAAAATGTTTACTAAATTAAATTGTAGTATGA -
- ATTGTTATAAATAATGAGGGAAAACAATTTACACATAGCAAATTTAAAAA -
- ATTGTTATAAATAATGAGGGAAAACAATTTACACATAGCAAATTTAAAAA -
- TTACTGTCATTTGATTTGTTAATATATTTTTCTCTTTAGTGGGAAATTAA -
- TTACTGTCATTTGATTTGTTAATATATTTTTCTCTTTAGTGGGAAATTAA -
- ATTTTAAAAAATTCCCTTTCGACTGTAGAACAAATAGGAATTTGGCCTGT -

FIGURE 50B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CTTTGTTGTAGGCCTAATGAGAAAAGGTTGAAGATAAAGTTCTGGTACTC
CTTTGTTGTAGGCCTAATGAGAAAAGGTTGAAGATAAAGTTCTGGTACTC
ATTTAAGTGTAATATTGAAAATTGATATTACCGAATCTGGAACAACCAAT
ATTTAAGTGTAATATTGAAAATTGATATTACCGAATCTGGAACAACCAAT
TTAAAATAAGGAAAGAAAGACACTGTGTTTTCT
TTAAAATAAGGAAAGAAAGACACTGTGTTTTCT

FIGURE 51A

```
                10         20         30         40         50         60
                |          |          |          |          |          |
  1 AGAAAACACA GTGTCTTTCT TTCCTTATTT TAAATTGGTT GTTCCAGATT CGGTAATATC
    TCTTTTGTGT CACAGAAAGA AAGGAATAAA ATTTAACCAA CAAGGTCTAA GCCATTATAG

 61 AATTTTCAAT ATTACACTTA AATGAGTACC AGAACTTTAT CTTCAACCTT TTCTCATTAG
    TTAAAAGTTA TAATGTGAAT TTACTCATGG TCTTGAAATA GAAGTTGGAA AAGAGTAATC

121 GCCTACAACA AAGGACATCT CGGATAGAAT TTCCCTTTTC TTTTTGCTAC TATAAGCTCT
    CGGATGTTGT TTCCTGTAGA GCCTATCTTA AAGGGAAAAG AAAAACGATG ATATTCGAGA

181 AAAAATCCTC AGAACATCAG ATTTAGAAAT GTTCTTATTA GTGGTAGTGA GCATTTGCTA
    TTTTTAGGAG TCTTGTAGTC TAAATCTTTA CAAGAATAAT CACCATCACT CGTAAACGAT

241 TTTCCTACCA CTAGCTTACA AATATAATAA GCAAGTAGAC CCCACAGGCC AAATTCCTAT
    AAAGGATGGT GATCGAATGT TTATATTATT CGTTCATCTG GGGTGTCCGG TTTAAGGATA

301 TTGTTCTACA GTCGAAAGGG AATTTTTTAA AATTTAATTT CCCACTAAAG AGAAAAATAT
    AACAAGATGT CAGCTTTCCC TTAAAAAATT TTAAATTAAA GGGTGATTTC TCTTTTTATA

361 ATTAACAAAT CAAATGACAG TAATTTTTAA ATTTGCTATG TGTAAATTGT TTTCCCTCAT
    TAATTGTTTA GTTTACTGTC ATTAAAAATT TAAACGATAC ACATTTAACA AAAGGGAGTA

421 TATTTATAAC AATTCATACT ACAATTTAAT TTAGTAAACA TTTTTGTAGA AAATATTTAA
    ATAAATATTG TTAAGTATGA TGTTAAATTA AATCATTTGT AAAAACATCT TTTATAAATT
```

FIGURE 51B

```
481 AACAAAGATA CTGAAAGTTA ATATNAAACC CAGTGCATGC TTCTTGTAGG CCACAGCCAT
    TTGTTTCTAT GACTTTCAAT TATANTTTGG GTCACGTACG AAGAACATCC GGTGTCGGTA

541 AACCTGTAAG CACAGAAAAA TTTGTTCTGT TACTCTAAAC ATCTACACTG GCCAAATTCC
    TTGGACATTC GTGTCTTTTT AAACAAGACA ATGAGATTTG TAGATGTGAC CGGTTTAAGG

601 AATGCTCGAA TTTAACCCCG GGATATAACC TAGTAAATGT GTCCTCTCTG TAAGGTGGGC
    TTACGAGCTT AAATTGGGGC CCTATATTGG ATCATTTACA CAGGAGAGAC ATTCCACCCG

661 ATGTCACAGA ATACAAGAAA ATAATGGTAT TCATAAACTT TTAAGAAAAT GATTCTACAC
    TACAGTGTCT TATGTTCTTT TATTACCATA AGTATTTCAA AATTCTTTTA CTAAGATGTG

721 ATGTAAAACC CACTATAACT TTTTACATTG GGGGAGAGAA AAAAAGAGAT AATTTTTACC
    TACATTTTGG GTGATATTGA AAAATGTAAC CCCCTCTCTT TTTTTCTCTA TTAAAAATGG

781 TT
    AA
```

FIGURE 52A

```
                10          20          30          40          50          60
                |           |           |           |           |           |
  1 GATGCTATTT GGGCAATTTC TTATTGACAG TTTTGAAATG TTAGGCTTTT ATCTCCATTT
    CTACGATAAA CCCGTTAAAG AATAACTGTC AAAACTTTAC AATCCGAAAA TAGAGGTAAA

 61 TTTAGTACTT AAATTTTCCA ACATGGGTGT TGCTTGTTAT TTTATCAGTA TAAAATAGAA
    AAATCATGAA TTTAAAAGGT TGTACCCACA ACGAACAATA AAATAGTCAT ATTTTATCTT

121 GAGTGGTTCT GTTCTGGAAT TTAGTATATA CATGAGTATC TAGTGTATGT CAGCCATGAA
    CTCACCAAGA CAAGACCTTA AATCATATAT GTACTCATAG ATCACATACA GTCGGTACTT

181 AATGAACCTT TCAGATGTTT AACTTCAGGG AACCTAATTG AGTCATTGCT CCAGACATTG
    TTACTTGGAA AGTCTACAAA TTGAAGTCCC TTGGATTAAC TCAGTAACGA GGTCTGTAAC

241 TTGCTTTGAA CCCACTATAT TNNNNNNNCT CGGGCAATGA CTCAGTGTGG CAAGGATACT
    AACGAAACTT GGGTGATATA ANNNNNNNGA GCCCGTTACT GAGTCACACC GTTCCTATGA

301 ACTGCAGGCC TGTTTCTGGA AGGCACTGGA CTCCTCTGAT GCAAACTTTG GCCAGGGACT
    TGACGTCCGG ACAAAGACCT TCCGTGACCT GAGGAGACTA CGTTTGAAAC CGGTCCCTGA

361 CCTTGATAGC TCTTAAATAG ATGCTGCACC AACACTCTCT TTCTTTTCTC TCTTTTTCTT
    GGAACTATCG AGAATTTATC TACGACGTGG TTGTGAGAGA AAGAAAAGAG AGAAAAAGAA
```

FIGURE 52B

```
421 TATTCAATAT TAGACTACAA GCAGTCTAAG GACTTCTCAG GGTTTCTAGC TCTCTCTCAT
    ATAAGTTATA ATCTGATGTT CGTCAGATTC CTGAAGAGTC CCAAAGATCG AGAGAGAGTA

481 TTCACACATG CTTTCCTAGT AATCTCTACT CATATATCTT ACTGCTACGC TGGGGCCAGA
    AAGTGTGTAC GAAAGGATCA TTAGAGATGA GTATATAGAA TGACGATGCG ACCCCGGTCT

541 TAACNNNNNN CTTCCATTTT GTTTTTATCT CTATTCTTCT TCCCCTTCTG CTTTCATTAT
    ATTGNNNNNN GAAGGTAAAA CAAAAATAGA GATAAGAAGA AGGGGAAGAC GAAAGTAATA

601 TGAAACTTTC TGCTTTCATT ATTGAAACTT TCCCAGATTT GTTCTGCTTA ACCTGGCATT
    ACTTTGAAAG ACGAAAGTAA TAACTTTGAA AGGGTCTAAA CAAGACGAAT TGGACCGTAA

661 GGAACTGTTT CCTCTTCCCT GTGCTGCTTT CTCCCATTGC CATGTCCTTT TTTTTTTTTT
    CCTTGACAAA GGAGAAGGGA CACGACGAAA GAGGGTAACG GTACAGGAAA AAAAAAAAAA

721 TTTTTTTTTT TGAGACAGTG TCACTCTGTT GCCCAGGCTG GAGTGCAATG GTGCAATCTT
    AAAAAAAAAA ACTCTGTCAC AGTGAGACAA CGGGTCCGAC CTCACGTTAC CACGTTAGAA
```

FIGURE 52C

```
781 GGCCACTGCA ACCCCCGCCT CCCGGGTTCA AGTGATTCTC CTGCCTCAGC CTCCTGAGTA
    CCGGTGACGT TGGGGGCGGA GGGCCCAAGT TCACTAAGAG GACGGAGTCG GAGGACTCAT

841 GCTGGGATTA CAGGTGCCCA CCACTATGCC CGGCTGATTT TTGTATTTTT AGTAGAGATN
    CGACCCTAAT GTCCACGGGT GGTGATACGG GCCGACTAAA AACATAAAAA TCATCTCTAN

901 NNNNNNNTTT CACCATNGCT GATCAGGCTG GTCTCGAACT CCTGACCGCA GTGANTCCGC
    NNNNNNNAAA GTGGTANCGA CTAGTCCGAC CAGAGCTTGA GGACTGGCGT CACTNAGGCG

961 CCTCCTTGGC CTCCCAAAGT GCTGAGATTA CAGGCATGAG TCACTGCGNC CAGCCACCAT
    GGAGGAACCG GAGGGTTTCA CGACTCTAAT GTCCGTACTC AGTGACGCNG GTCGGTGGTA

1021 TATTCTCTAG AGGTGAGAGA ACACTGGCTC TTCTAACAAG TTGAAATTTG ATAGAGACC
     ATAAGAGATC TCCACTCTCT TGTGACCGAG AAGATTGTTC AACTTTAAAC TATCTCTGG
```

FIGURE 53A

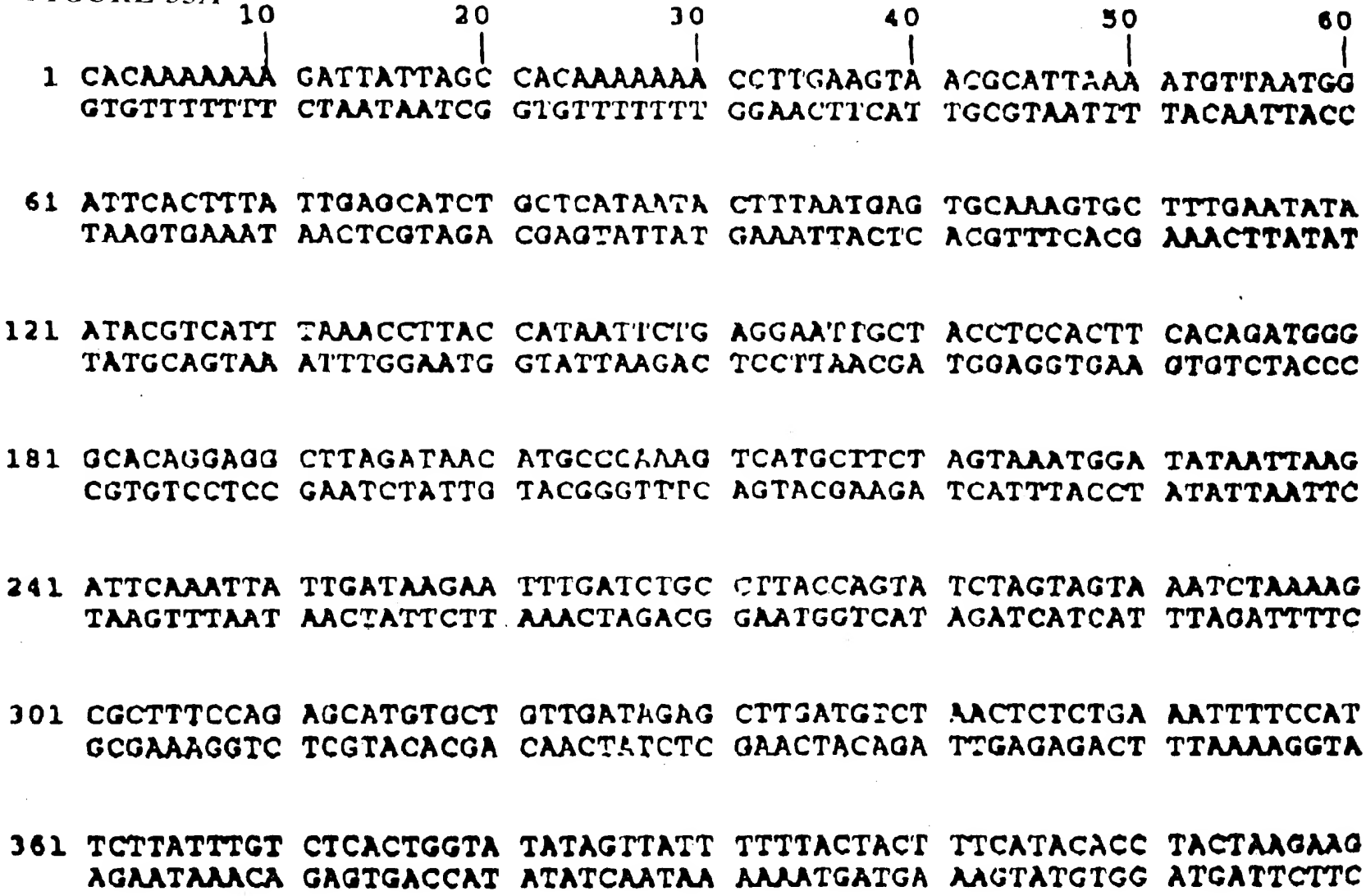

10 20 30 40 50 60
1 CACAAAAAAA GATTATTAGC CACAAAAAAA CCTTGAAGTA ACGCATTAAA ATGTTAATGG
GTGTTTTTTT CTAATAATCG GTGTTTTTTT GGAACTTCAT TGCGTAATTT TACAATTACC
61 ATTCACTTTA TTGAGCATCT GCTCATAATA CTTTAATGAG TGCAAAGTGC TTTGAATATA
TAAGTGAAAT AACTCGTAGA CGAGTATTAT GAAATTACTC ACGTTTCACG AAACTTATAT
121 ATACGTCATT TAAACCTTAC CATAATTCTG AGGAATTGCT ACCTCCACTT CACAGATGGG
TATGCAGTAA ATTTGGAATG GTATTAAGAC TCCTTAACGA TGGAGGTGAA GTGTCTACCC
181 GCACAGGAGG CTTAGATAAC ATGCCCAAAG TCATGCTTCT AGTAAATGGA TATAATTAAG
CGTGTCCTCC GAATCTATTG TACGGGTTTC AGTACGAAGA TCATTTACCT ATATTAATTC
241 ATTCAAATTA TTGATAAGAA TTTGATCTGC CTTACCAGTA TCTAGTAGTA AATCTAAAAG
TAAGTTTAAT AACTATTCTT AAACTAGACG GAATGGTCAT AGATCATCAT TTAGATTTTC
301 CGCTTTCCAG AGCATGTGCT GTTGATAGAG CTTGATGTCT AACTCTCTGA AATTTTCCAT
GCGAAAGGTC TCGTACACGA CAACTATCTC GAACTACAGA TTGAGAGACT TTAAAAGGTA
361 TCTTATTTGT CTCACTGGTA TATAGTTATT TTTTACTACT TTCATACACC TACTAAGAAG
AGAATAAACA GAGTGACCAT ATATCAATAA AAAATGATGA AAGTATGTGG ATGATTCTTC

FIGURE 53B

```
421 ACAGGAGGAT CAAAGATAGG ATTTCATTTA GAATGCCTAA AGCTTCACGT ATTTTAATTC
    TGTCCTCCTA GTTTCTATCC TAAAGTAAAT CTTACGGATT TCGAAGTGCA TAAAATTAAG

481 AGAATAAGAT TCAGGCAGAC CACCAGTATA TGCCATGGTC CCTGGTTATC TTTCAGCAGG
    TCTTATTCTA AGTCCGTCTG GTGGTCATAT ACGGTACCAG GGACCAATAG AAAGTCGTCC

541 TGACCGAGAA AGAAAACATG GTAATGTTTA TGAAATGGTG GGTTCTTGTA GTTTCACTTC
    ACTGGCTCTT TCTTTTGTAC CATTACAAAT ACTTTACCAC CCAAGAACAT CAAAGTGAAG

601 AACATATCTG CCTTTACTGT ATTAAGATGA TGGATTAACT TATTCTTGAT ATGGGCATGT
    TTGTATAGAC GGAAATGACA TAATTCTACT ACCTAATTGA ATAAGAACTA TACCCGTACA

661 AAAACAATAT ACTTTTACTA AACAGCTACA GAGAGACAAA TGTGTTTCCA GACAAACTTA
    TTTTGTTATA TGAAAATGAT TTGTCGATGT CTCTCTGTTT ACACAAAGGT CTGTTTGAAT

721 AGAGACTGAG TGTTCAAACT GAATAATCTC GACCTTAATT GTAACTATAT TTTATGAAAT
    TCTCTGACTC ACAAGTTTGA CTTATTAGAG CTGGAATTAA CATTGATATA AAATACTTTA
```

FIGURE 53C

```
781  CCAGCTGTAA GCCAAAAACA GACTTCTTTG GGCCTACCAC GGGCATTTTG TTCCTGTTAN
     GGTCGACATT CCGTTTTTGT CTGAAGAAAC CCGGATGGTG CCCGTAAAAC AAGGACAATN

841  NNNTACTCCA AACCTTAAAC CCACGTCCAC TTAAATAATG GCCTGGAAAT AAATGTCATT
     NNNATGAGGT TTGGAATTTG GGTGCAGGTG AATTTATTAC CGGACCTTTA TTTACAGTAA

901  ATCTGATATT ATACTGAGAT GTTTAGTTAT GAAATCAAAA GTGGAGAATT TCAATCTGTC
     TAGACTATAA TATGACTCTA CAAATCAATA CTTTAGTTTT CACCTCTTAA AGTTAGACAG

961  CTGTAAGCTT TCTCTGCGGT CACGACCCTC ATGCACTCAG GCTGTGCGGT GCAGCATGCT
     GACATTCGAA AGAGACGCCA GTGCTGGGAG TACGTGAGTC CGACACGCCA CGTCGTACGA

1021 CTGTCATGTC TGTTTTCTTC TGCCTGTACA CGGGTGGTTG TTCCTGTCTA CCTGTTTGAG
     GACAGTACAG ACAAAAGAAG ACGGACATGT GCCCACCAAC AAGGACAGAT GGACAAACTC

1081 GAAATATGAA TACGTNNNNN NCTAGAATCT ACTGCACATG CAATAAGGAA ACAATCAGTA
     CTTTATACTT ATGCANNNNN NGATCTTAGA TGACGTGTAC GTTATTCCTT TGTTAGTCAT

1141 AGAATCACTT TCTCGTGGAA AATTCATTAG AATTAACATC TCGTTTTAAA ATGCTCTATC
     TCTTAGTGAA AGAGCACCTT TTAAGTAATC TTAATTGTAG AGCAAAATTT TACGAGATAG
```

FIGURE 53D

```
1201 AAAGTGTAAA TAATTCCTCT CTCTTTTCCC TTTTTCACTA AGGAGTTTGT ATATTAAACA
     TTTCACATTT ATTAAGGAGA GAGAAAAGGG AAAAAGTGAT TCCTCAAACA TATAATTTGT

1261 GAATTTCAAG TAATGTATTA TAAATTTATT TAANNTATTT ACAATAAAAT GCCACGTATA
     CTTAAAGTTC ATTACATAAT ATTTAAATAA ATTNNATAAA TGTTATTTTA CGGTGCATAT

1321 AGCATCAAGC AACATGANNN NNNCATTGGT AGAAAGCACA ATACATAGTC AAAACAGCAG
     TCGTAGTTCG TTGTACTNNN NNNGTAACCA TCTTTCGTGT TATGTATCAG TTTTGTCGTC

1381 AGTATTAAAT AAACAGAAAA TTTGCAAAAG GCAAGTAAAG AATATACATA TACTTAATTA
     TCATAATTTA TTTGTCTTTT AAACGTTTTC CGTTCATTTC TTATATGTAT ATGAATTAAT

1441 TACATAAAAT ATTGATACAG GAGGTAGAAA GAAATTTAGT AAGCAGATAA TGGGGGCAAC
     ATGTATTTTA TAACTATGTC CTCCATCTTT CTTTAAATCA TTCGTCTATT ACCCCCGTTG

1501 AGAGTCCTCA GCAGAGCTTC CCTTCTAACA AAAAGCAGCC CAATAAATTA TTTTTTTTTT
     TCTCAGGAGT CGTCTCGAAG GGAAGATTGT TTTTCGTCGG GTTATTTAAT AAAAAAAAAA

1561 CTAACAAAAA GCAGCCTGAA AAATCGAGCT GCAAACATAG ATTAGCAATC GGCTGAAAGT
```

FIGURE 53E

```
     GATTGTTTTT CGTCGGACTT TTTAGCTCGA CGTTTGTATC TAATCGTTAG CCGACTTTCA

1621 GCGGGAGAAT GCTGGCAGCT GTGCCAATAG TAAAGGGCTA CCTGGAGCCG GGCGCGTGGC
     CGCCCTCTTA CGACCGTCGA CACGGTTATC ATTTCCCGAT GGACCTCGGC CCGCGCACCG

1681 TCACGCTGTA ATCCCAGCAC TTTGGGAGGG CGAGGCAACG CGGATCACCT GAGGTCGGGA
     AGTGCGACAT TAGGGTCGTG AAACCCTCCC GCTCCGTTGC GCCTAGTGGA CTCCAGCCCT

1741 GTTTGAGATC AGCCCGACCA ACATGGAGAA ACCCCGTCTC TACTAAAAAA AAAAAAAAAA
     CAAACTCTAG TCGGGCTGGT TGTACCTCTT TGGGGCAGAG ATGATTTTTT TTTTTTTTTT

1801 AAAGGCAAAA AATGAGCCGG GCATGGTGGC ACATGCCTTG CACATCCCAG CTGAGGCAGG
     TTTCCGTTTT TTACTCGGCC CGTACCACCG TGTACGGAAC GTGTAGGGTC GACTCCGTCC

1861 AGAATTCACT TGAACCTGGG AGGTAGAGAT TGCGGTGAAG CGAGATCACG TCATTGCACT
     TCTTAAGTGA ACTTGGACCC TCCATCTCTA ACGCCACTTC GCTCTAGTGC AGTAACGTGA

1921 CCAGCCTGGG CAAAAAGAGC AAAACTTAGT CTCAAAAAAA AAAANNCAAA GAAAAAA
     GGTCGGACCC GTTTTTCTCG TTTTGAATCA GAGTTTTTTT TTTTNNGTTT CTTTTTT
```

FIGURE 54

Genomic Organization of PSM Gene

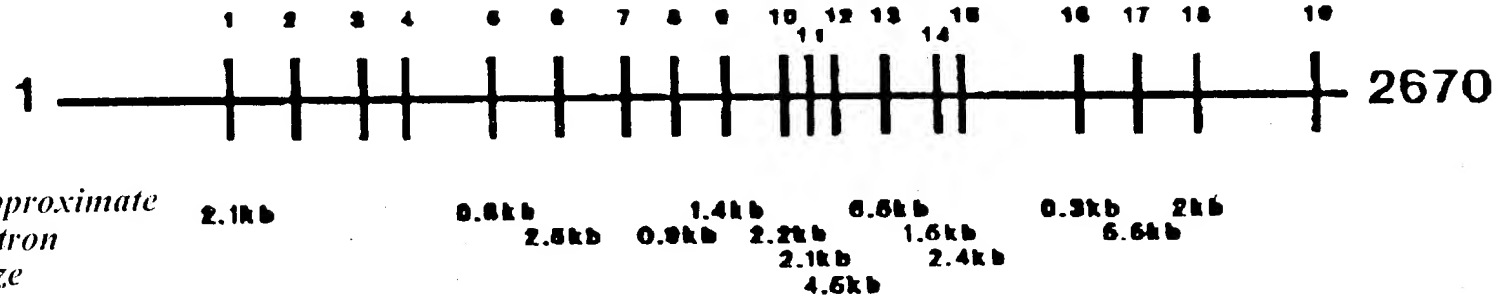


FIGURE 55A

```
          10          20          30          40
     *    *    *    *    *    *    *    *    *    *
CTC AAA AGG GGC CGG ATT TCC TTC TCC TGG AGG CAG ATG TTG CCT CTC

50          60          70          80          90
 *    *    *    *    *    *    *    *    *    *
TCT CTC GCT CGG ATT GGT TCA GTG CAC TCT AGA AAC ACT GCT GTG GTG

  100         110         120         130         140
    *    *    *    *    *    *    *    *    *
GAG AAA CTG GAC CCC AGG GTG GTT TAT AAA ATC CTC CAA TGA AGC TAC

    150         160         170         180         190
*    *    *    *    *    *    *    *    *    *
TAA CAT TAC TCC AAA GCA TAA TAT GAA AGC ATT TTT GGA TGA ATT GAA

                                  Met Lys Ala Phe Leu Asp Glu Leu Lys>

      200         210         220         230         240
  *    *    *    *    *    *    *    *    *    *
AGC TGA GAA CAT CAA GAA GTT CTT ATA TAA TTT TAC ACA GAT ACC ACA

 Ala Glu Asn Ile Lys Lys Phe Leu Tyr Asn Phe Thr Gln Ile Pro His>
```

FIGURE 55B

```
          250         260         270         280
     *    *    *    *    *    *    *    *    *
TTT AGC AGG AAC AGA ACA AAA CTT TCA GCT TGC AAA GCA AAT TCA ATC

 Leu Ala Gly Thr Glu Gln Asn Phe Gln Leu Ala Lys Gln Ile Gln Ser>

290         300         310         320         330
  *    *    *    *    *    *    *    *    *    *
CCA GTG GAA AGA ATT TGG CCT GGA TTC TGT TGA GCT AGC ACA TTA TGA

 Gln Trp Lys Glu Phe Gly Leu Asp Ser Val Glu Leu Ala His Tyr Asp>

  340         350         360         370         380
     *    *    *    *    *    *    *    *    *
TGT CCT GTT GTC CTA CCC AAA TAA GAC TCA TCC CAA CTA CAT CTC AAT

 Val Leu Leu Ser Tyr Pro Asn Lys Thr His Pro Asn Tyr Ile Ser Ile>

    390         400         410         420         430
*    *    *    *    *    *    *    *    *    *
AAT TAA TGA AGA TGG AAA TGA GAT TTT CAA CAC ATC ATT ATT TGA ACC

 Ile Asn Glu Asp Gly Asn Glu Ile Phe Asn Thr Ser Leu Phe Glu Pro>

       440         450         460         470         480
  *    *    *    *    *    *    *    *    *    *
ACC TCC TCC AGG ATA TGA AAA TGT TTC GGA TAT TGT ACC ACC TTT CAG

 Pro Pro Pro Gly Tyr Glu Asn Val Ser Asp Ile Val Pro Pro Phe Ser>
```

FIGURE 55C

```
          490         500         510         520
      *   *   *   *   *   *   *   *   *   *
TGC TTT CTC TCC TCA AGG AAT GCC AGA GGG CGA TCT AGT GTA TGT TAA

 Ala Phe Ser Pro Gln Gly Met Pro Glu Gly Asp Leu Val Tyr Val Asn>

530         540         550         560         570
  *   *   *   *   *   *   *   *   *   *
CTA TGC ACG AAC TGA AGA CTT CTT TAA ATT GGA ACG GGA CAT GAA AAT

 Tyr Ala Arg Thr Glu Asp Phe Phe Lys Leu Glu Arg Asp Met Lys Ile>

 580         590         600         610         620
    *   *   *   *   *   *   *   *   *   *
CAA TTG CTC TGG GAA AAT TGT AAT TGC CAG ATA TGG GAA AGT TTT CAG

 Asn Cys Ser Gly Lys Ile Val Ile Ala Arg Tyr Gly Lys Val Phe Arg>

    630         640         650         660         670
*   *   *   *   *   *   *   *   *   *   *
AGG AAA TAA GGT TAA AAA TGC CCA GCT GGC AGG GGC CAA AGG AGT CAT

 Gly Asn Lys Val Lys Asn Ala Gln Leu Ala Gly Ala Lys Gly Val Ile>

      680         690         700         710         720
  *   *   *   *   *   *   *   *   *   *   *
TCT CTA CTC CGA CCC TGC TGA CTA CTT TGC TCC TGG GGT GAA GTC CTA

 Leu Tyr Ser Asp Pro Ala Asp Tyr Phe Ala Pro Gly Val Lys Ser Tyr>
```

FIGURE 55D

```
          730         740         750         760
      *   *   *   *   *   *   *   *   *   *   *
TCC AGA TGG TTG GAA TCT TCC TGG AGG TGG TGT CCA GCG TGG AAA TAT

 Pro Asp Gly Trp Asn Leu Pro Gly Gly Gly Val Gln Arg Gly Asn Ile>

770         780         790         800         810
  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CCT AAA TCT GAA TGG TGC AGG AGA CCC TCT CAC ACC AGG TTA CCC AGC

 Leu Asn Leu Asn Gly Ala Gly Asp Pro Leu Thr Pro Gly Tyr Pro Ala>

  820         830         840         850         860
    *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
AAA TGA ATA TGC TTA TAG GCG TGG AAT TGC AGA GGC TGT TGG TCT TCC

 Asn Glu Tyr Ala Tyr Arg Arg Gly Ile Ala Glu Ala Val Gly Leu Pro>

    870         880         890         900         910
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *
AAG TAT TCC TGT TCA TCC AAT TGG ATA CTA TGA TGC ACA GAA GCT CCT

 Ser Ile Pro Val His Pro Ile Gly Tyr Tyr Asp Ala Gln Lys Leu Leu>

      920         930         940         950         960
  *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
AGA AAA AAT GGG TGG CTC AGC ACC ACC AGA TAG CAG CTG GAG AGG AAG

 Glu Lys Met Gly Gly Ser Ala Pro Pro Asp Ser Ser Trp Arg Gly Ser>
```

FIGURE 55E

```
          970         980         990         1000
     *    *    *    *    *    *    *    *    *    *
TCT CAA AGT GCC CTA CAA TGT TGG ACC TGG CTT TAC TGG AAA CTT TTC

 Leu Lys Val Pro Tyr Asn Val Gly Pro Gly Phe Thr Gly Asn Phe Ser>

1010        1020        1030        1040        1050
   *    *    *    *    *    *    *    *    *    *
TAC ACA AAA AGT CAA GAT GCA CAT CCA CTC TAC CAA TGA AGT GAC AAG

 Thr Gln Lys Val Lys Met His Ile His Ser Thr Asn Glu Val Thr Arg>

 1060        1070        1080        1090        1100
    *    *    *    *    *    *    *    *    *
AAT TTA CAA TGT GAT AGG TAC TCT CAG AGG AGC AGT GGA ACC AGA CAG

 Ile Tyr Asn Val Ile Gly Thr Leu Arg Gly Ala Val Glu Pro Asp Arg>

   1110        1120        1130        1140        1150
*    *    *    *    *    *    *    *    *    *
ATA TGT CAT TCT GGG AGG TCA CCG GGA CTC ATG GGT GTT TGG TGG TAT

 Tyr Val Ile Leu Gly Gly His Arg Asp Ser Trp Val Phe Gly Gly Ile>

     1160        1170        1180        1190        1200
  *    *    *    *    *    *    *    *    *    *
TGA CCC TCA GAG TGG AGC AGC TGT TGT TCA TGA AAT TGT GAG GAG CTT

 Asp Pro Gln Ser Gly Ala Ala Val Val His Glu Ile Val Arg Ser Phe>

         1210        1220        1230        1240
     *    *    *    *    *    *    *    *    *
TGG AAC ACT GAA AAA GGA AGG GTG GAG ACC TAG AAG AAC AAT TTT GTT

 Gly Thr Leu Lys Lys Glu Gly Trp Arg Pro Arg Arg Thr Ile Leu Phe>
```

FIGURE 55F

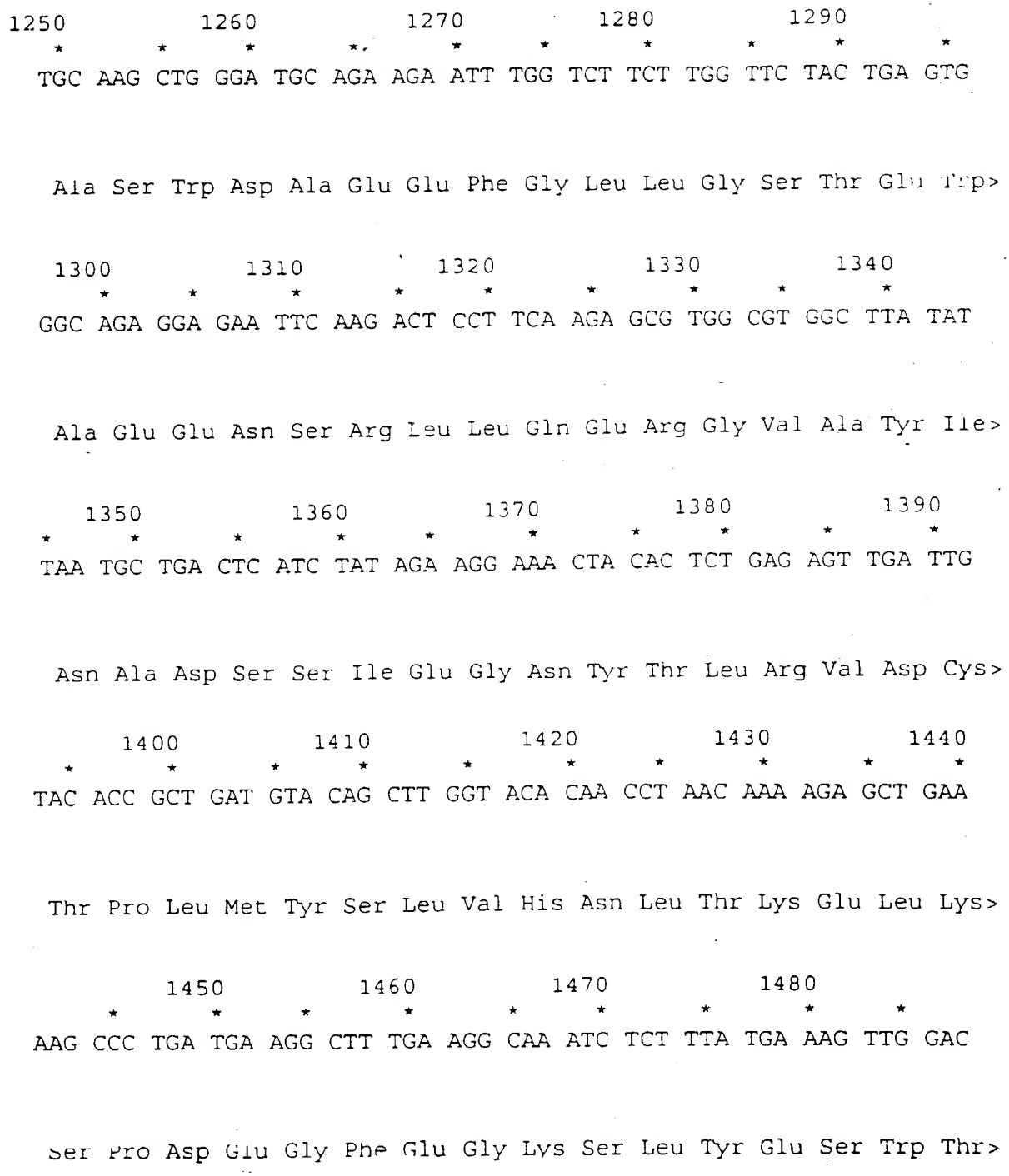

1250 1260 1270 1280 1290
TGC AAG CTG GGA TGC AGA AGA ATT TGG TCT TCT TGG TTC TAC TGA GTG
Ala Ser Trp Asp Ala Glu Glu Phe Gly Leu Leu Gly Ser Thr Glu Trp>
1300 1310 1320 1330 1340
GGC AGA GGA GAA TTC AAG ACT CCT TCA AGA GCG TGG CGT GGC TTA TAT
Ala Glu Glu Asn Ser Arg Leu Leu Gln Glu Arg Gly Val Ala Tyr Ile>
1350 1360 1370 1380 1390
TAA TGC TGA CTC ATC TAT AGA AGG AAA CTA CAC TCT GAG AGT TGA TTG
Asn Ala Asp Ser Ser Ile Glu Gly Asn Tyr Thr Leu Arg Val Asp Cys>
1400 1410 1420 1430 1440
TAC ACC GCT GAT GTA CAG CTT GGT ACA CAA CCT AAC AAA AGA GCT GAA
Thr Pro Leu Met Tyr Ser Leu Val His Asn Leu Thr Lys Glu Leu Lys>
1450 1460 1470 1480
AAG CCC TGA TGA AGG CTT TGA AGG CAA ATC TCT TTA TGA AAG TTG GAC
Ser Pro Asp Glu Gly Phe Glu Gly Lys Ser Leu Tyr Glu Ser Trp Thr>

FIGURE 55G

```
1490          1500          1510          1520          1530
  *     *     *     *     *     *     *     *     *     *
TAA AAA AAG TCC TTC CCC AGA GTT CAG TGG CAT GCC CAG GAT AAG CAA

 Lys Lys Ser Pro Ser Pro Glu Phe Ser Gly Met Pro Arg Ile Ser Lys>

 1540          1550          1560          1570          1580
    *     *     *     *     *     *     *     *     *     *
ATT GGG ATC TGG AAA TGA TTT TGA GGT GTT CTT CCA ACG ACT TGG AAT

 Leu Glv Ser Gly Asn Asp Phe Glu Val Phe Phe Gln Arg Leu Gly Ile>

   1590          1600          1610          1620          1630
*     *     *     *     *     *     *     *     *     *
TGC TTC AGG CAG AGC ACG GTA TAC TAA AAA TTG GGA AAC AAA CAA ATT

 Ala Ser Gly Arg Ala Arg Tyr Thr Lys Asn Trp Glu Thr Asn Lys Phe>

     1640          1650          1660          1670          1680
  *     *     *     *     *     *     *     *     *     *
CAG CGG CTA TCC ACT GTA TCA CAG TGT CTA TGA AAC ATA TGA GTT GGT

 Ser Gly Tyr Pro Leu Tyr His Ser Val Tyr Glu Thr Tyr Glu Leu Val>

         1690          1700          1710          1720
     *     *     *     *     *     *     *     *     *
GGA AAA GTT TTA TGA TCC AAT GTT TAA ATA TCA CCT CAC TGT GGC CCA

 Glu Lys Phe Tyr Asp Pro Met Phe Lys Tyr His Leu Thr Val Ala Gln>
```

FIGURE 55H

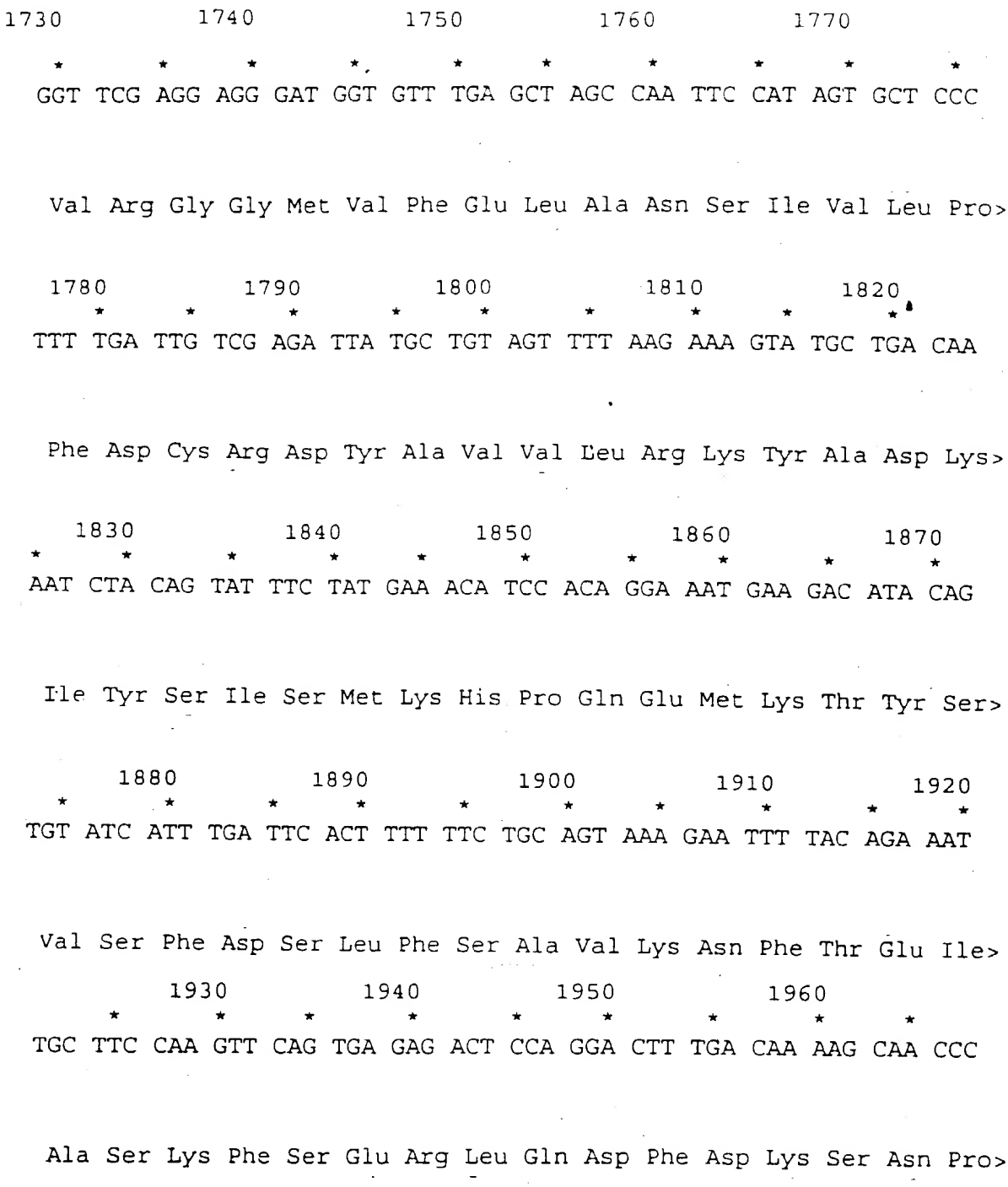

1730 1740 1750 1760 1770
GGT TCG AGG AGG GAT GGT GTT TGA GCT AGC CAA TTC CAT AGT GCT CCC
Val Arg Gly Gly Met Val Phe Glu Leu Ala Asn Ser Ile Val Leu Pro>
1780 1790 1800 1810 1820
TTT TGA TTG TCG AGA TTA TGC TGT AGT TTT AAG AAA GTA TGC TGA CAA
Phe Asp Cys Arg Asp Tyr Ala Val Val Leu Arg Lys Tyr Ala Asp Lys>
1830 1840 1850 1860 1870
AAT CTA CAG TAT TTC TAT GAA ACA TCC ACA GGA AAT GAA GAC ATA CAG
Ile Tyr Ser Ile Ser Met Lys His Pro Gln Glu Met Lys Thr Tyr Ser>
1880 1890 1900 1910 1920
TGT ATC ATT TGA TTC ACT TTT TTC TGC AGT AAA GAA TTT TAC AGA AAT
Val Ser Phe Asp Ser Leu Phe Ser Ala Val Lys Asn Phe Thr Glu Ile>
1930 1940 1950 1960
TGC TTC CAA GTT CAG TGA GAG ACT CCA GGA CTT TGA CAA AAG CAA CCC
Ala Ser Lys Phe Ser Glu Arg Leu Gln Asp Phe Asp Lys Ser Asn Pro>

FIGURE 55I

```
1970          1980          1990          2000          2010
   *     *     *     *     *     *     *     *     *     *
AAT AGT ATT AAG AAT GAT GAA TGA TCA ACT CAT GTT TCT GGA AAG AGC

 Ile Val Leu Arg Met Met Asn Asp Gln Leu Met Phe Leu Glu Arg Ala>

2020          2030          2040          2050          2060
   *     *     *     *     *     *     *     *     *
ATT TAT TGA TCC ATT AGG GTT ACC AGA CAG GCC TTT TTA TAG GCA TGT

 Phe Ile Asp Pro Leu Gly Leu Pro Asp Arg Pro Phe Tyr Arg His Val>

2070          2080          2090          2100          2110
*     *     *     *     *     *     *     *     *     *
CAT CTA TGC TCC AAG CAG CCA CAA CAA GTA TGC AGG GGA GTC ATT CCC

 Ile Tyr Ala Pro Ser Ser His Asn Lys Tyr Ala Gly Glu Ser Phe Pro>

2120          2130          2140          2150          2160
   *     *     *     *     *     *     *     *     *     *
AGG AAT TTA TGA TGC TCT GTT TGA TAT TGA AAG CAA AGT GGA CCC TTC

 Gly Ile Tyr Asp Ala Leu Phe Asp Ile Glu Ser Lys Val Asp Pro Ser>

2170          2180          2190          2200
   *     *     *     *     *     *     *     *     *
CAA GGC CTG GGG AGA AGT GAA GAG ACA GAT TTA TGT TGC AGC CTT CAC

 Lys Ala Trp Gly Glu Val Lys Arg Gln Ile Tyr Val Ala Ala Phe Thr>

2210          2220          2230          2240          2250
   *     *     *     *     *     *     *     *     *     *
AGT GCA GGC AGC TGC AGA GAC TTT GAG TGA AGT AGC CTA AGA GGA TTC

 Val Gln Ala Ala Ala Glu Thr Leu Ser Glu Val Ala
```

FIGURE 53J

```
2260          2270          2280          2290          2300
   *     *       *     *     *     *       *     *       *
TTT AGA GAA TCC GTA TTG AAT TTG TGT GGT ATG TCA CTC AGA AAG AAT

   2310          2320          2330          2340          2350
*     *       *     *     *     *     *       *     *       *
CGT AAT GGG TAT ATT GAT AAA TTT TAA AAT TGG TAT ATT TGA AAT AAA

      2360          2370          2380
  *       *     *     *     *       *     *
GTT GAA TAT TAT ATA TAA AAA AAA AAA AAA AAA AA
```

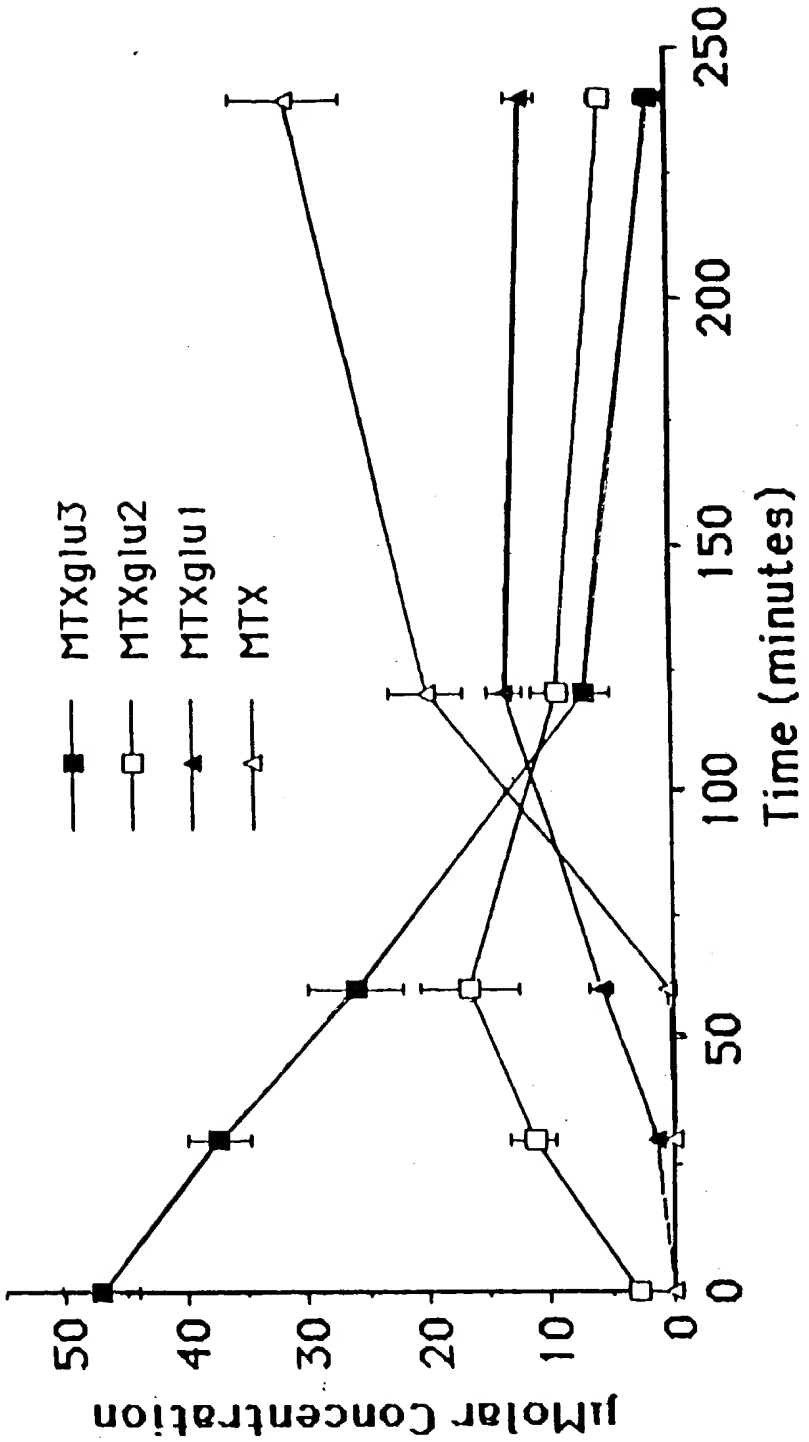

MTXglu3
MTXglu2
MTXglu1
MTX
50
40
30
20
10
0
0
50
100
150
200
250
Time (minutes)
μMolar Concentration

FIGURE 56

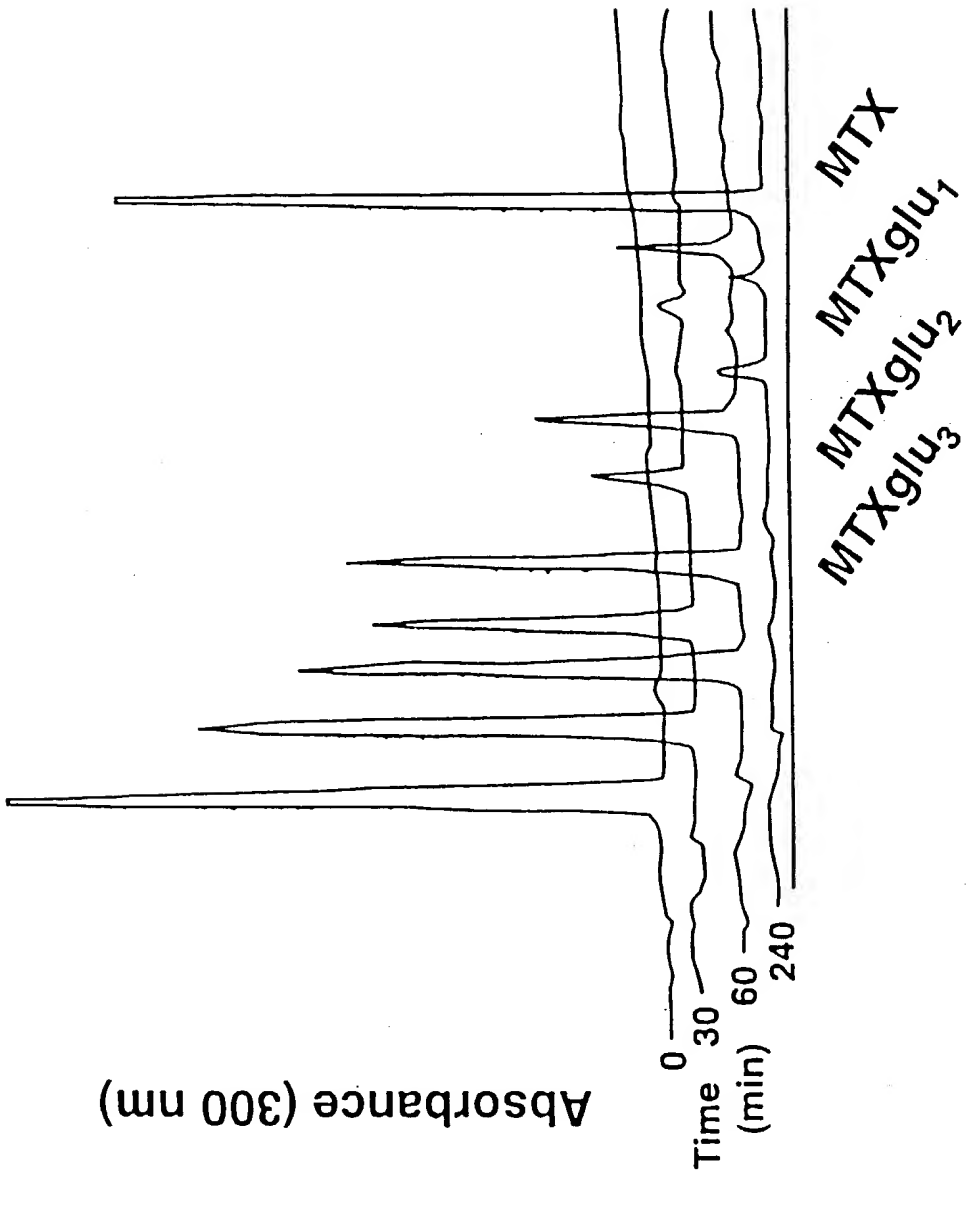
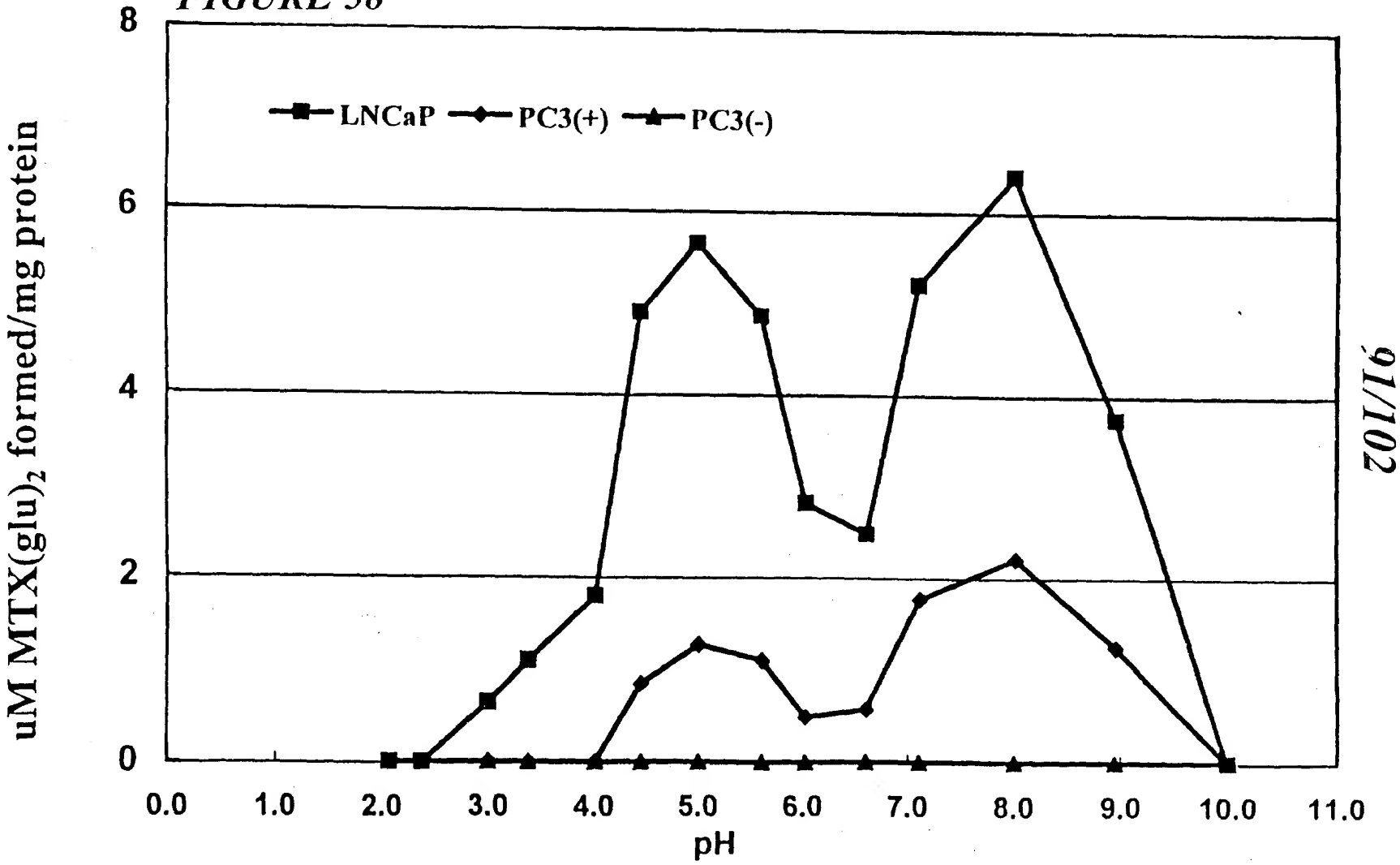

MTX
MTXglu1
MTXglu2
MTXglu3
Absorbance (300 nm)
Time (min)
0
30
60
240

FIGURE 57

FIGURE 58



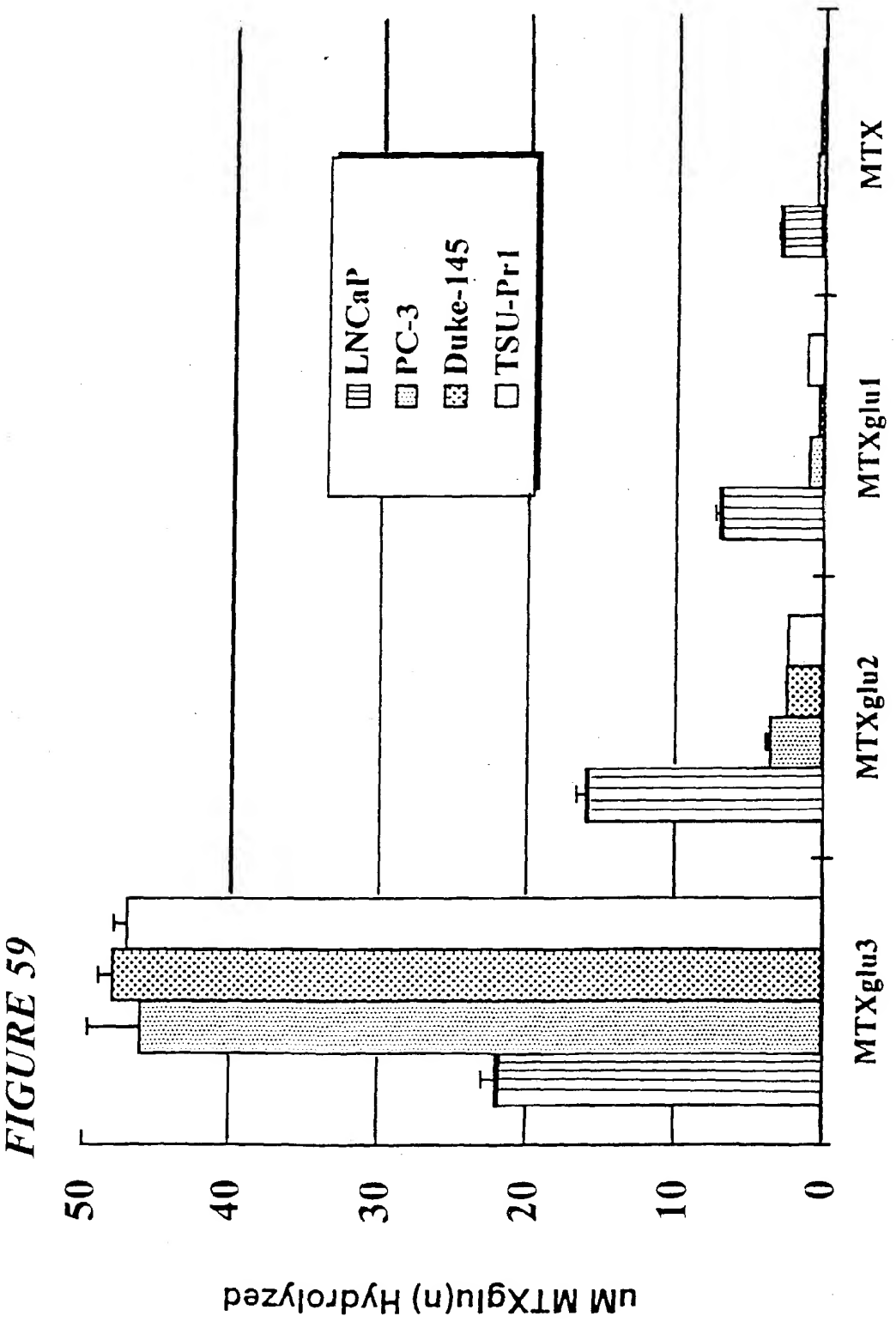

uM MTXglu(n) Hydrolyzed
50
40
30
20
10
0
MTXglu3
MTXglu2
MTXglu1
MTX
LNCaP
PC-3
Duke-145
TSU-Pr1

FIGURE 59

FIGURE 60A

FIGURE 60B

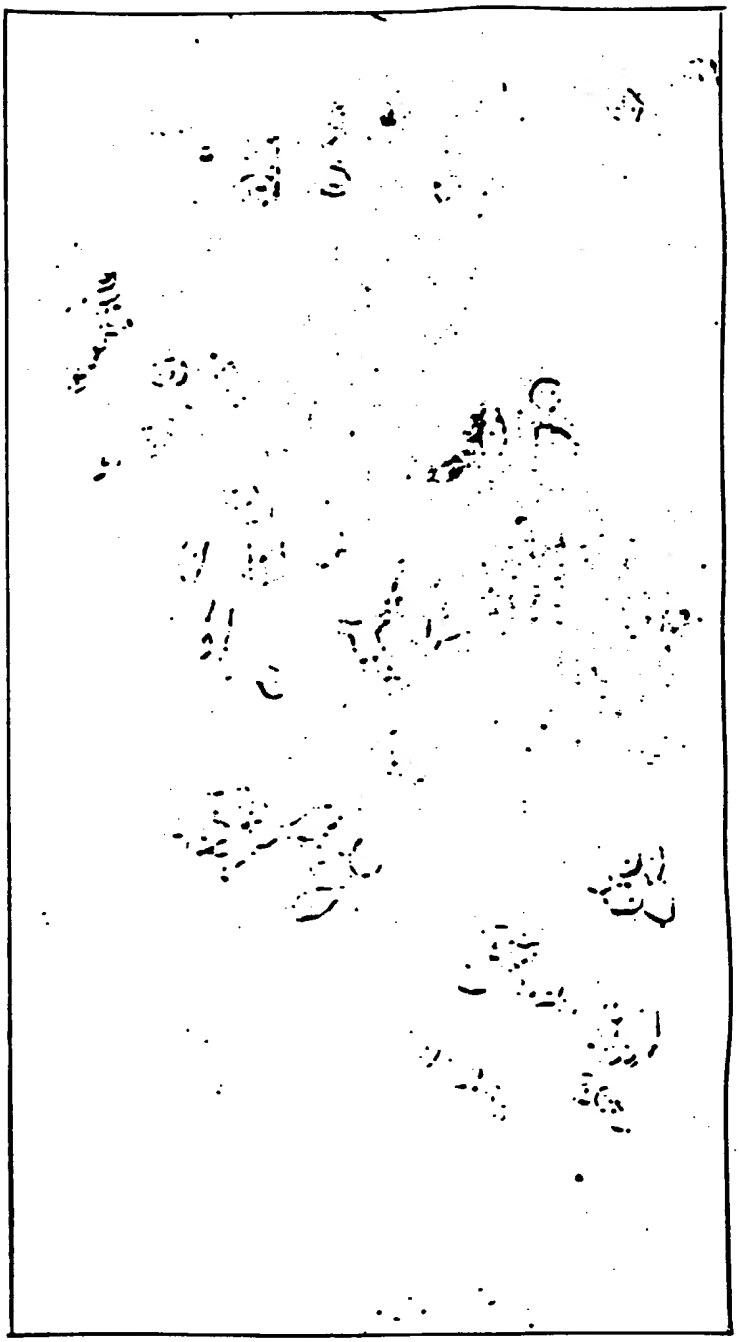


FIGURE 60C

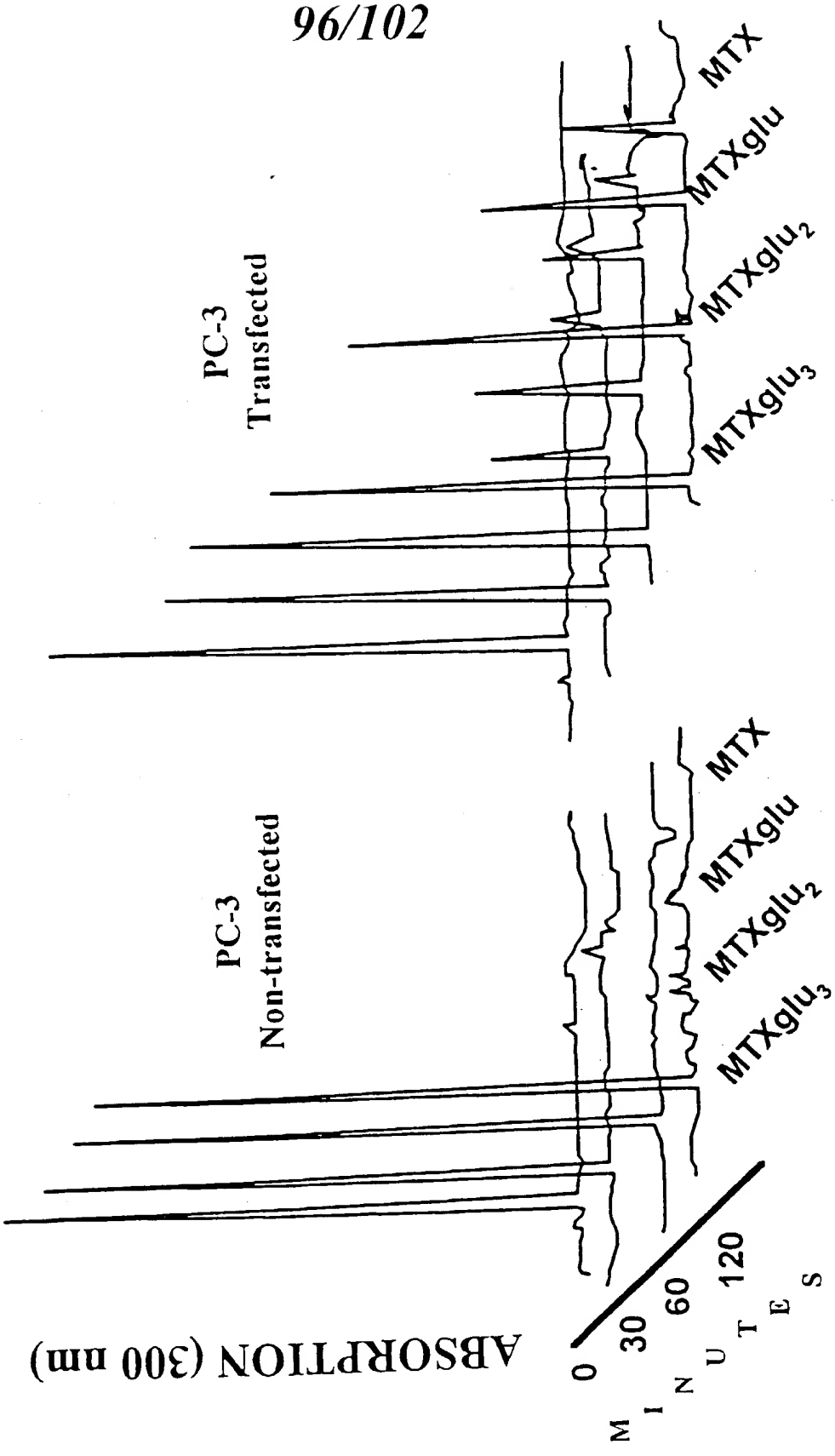
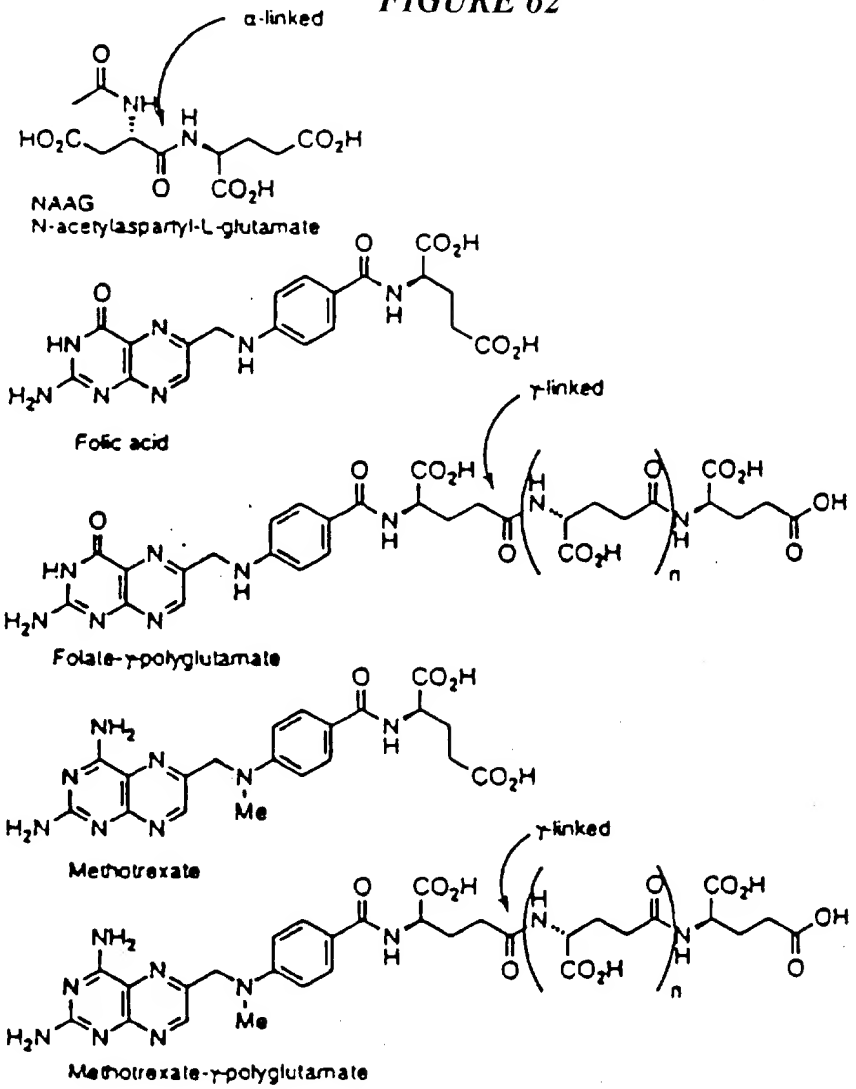

PC-3
Transfected
MTX
MTXglu
MTXglu2
MTXglu3
PC-3
Non-transfected
MTX
MTXglu
MTXglu2
MTXglu3
ABSORPTION (300 nm)
0
30
60
120
MINUTES

FIGURE 61

FIGURE 62


α-linked
NAAG
N-acetylaspartyl-L-glutamate
Folic acid
γ-linked
Folate-γ-polyglutamate
Methotrexate
γ-linked
Methotrexate-γ-polyglutamate

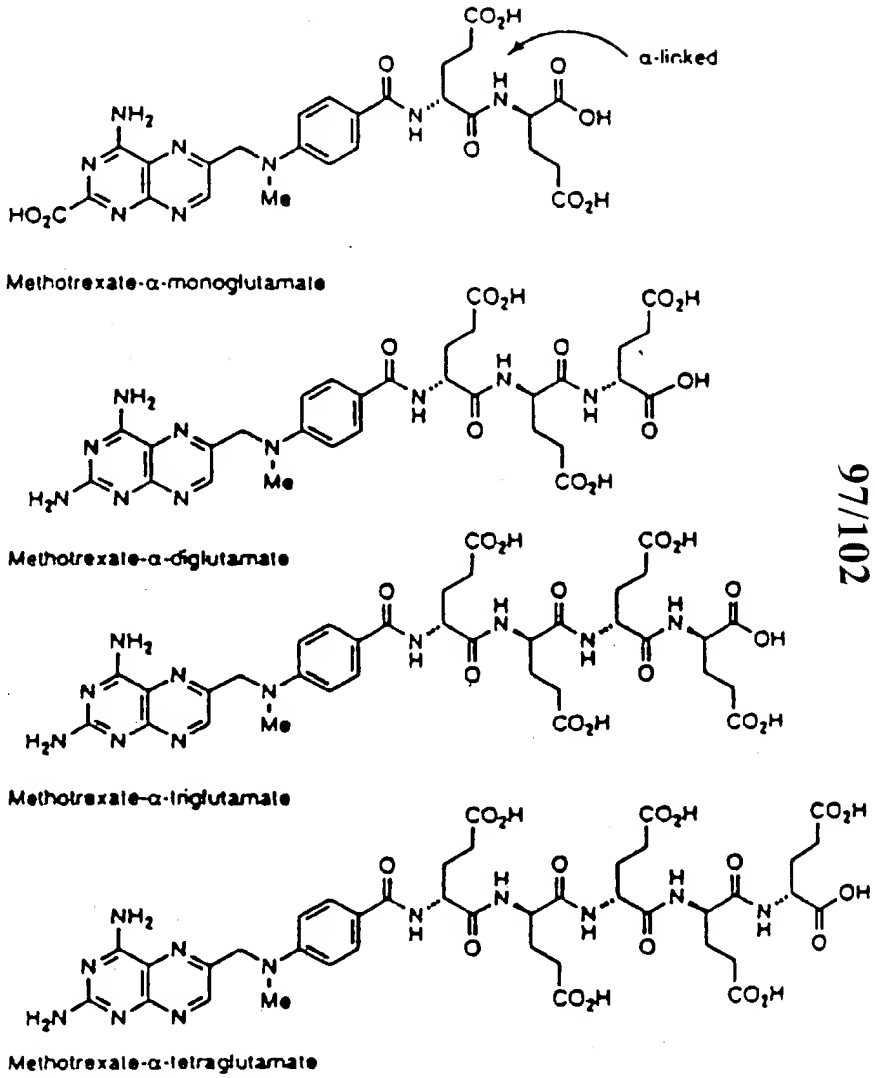

α-linked
Methotrexate-α-monoglutamate
Methotrexate-α-diglutamate
Methotrexate-α-triglutamate
Methotrexate-α-tetraglutamate

FIGURE 63A

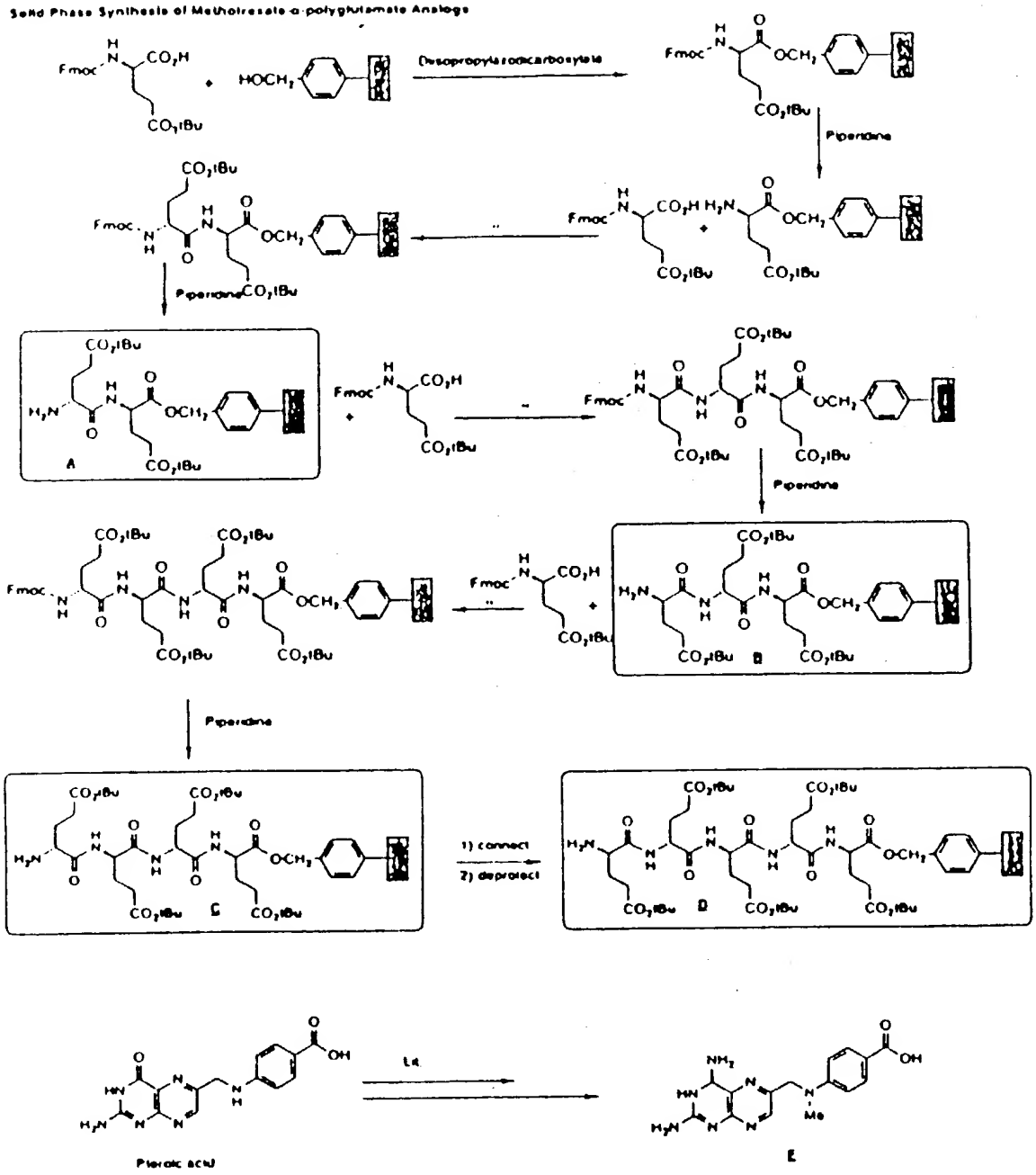

Solid Phase Synthesis of Methotrexate-α-polyglutamate Analogs
Diisopropylcarbodiimide
Piperidine
A
B
C
D
E
1) connect
2) deprotect
Pteroic acid

FIGURE 63B

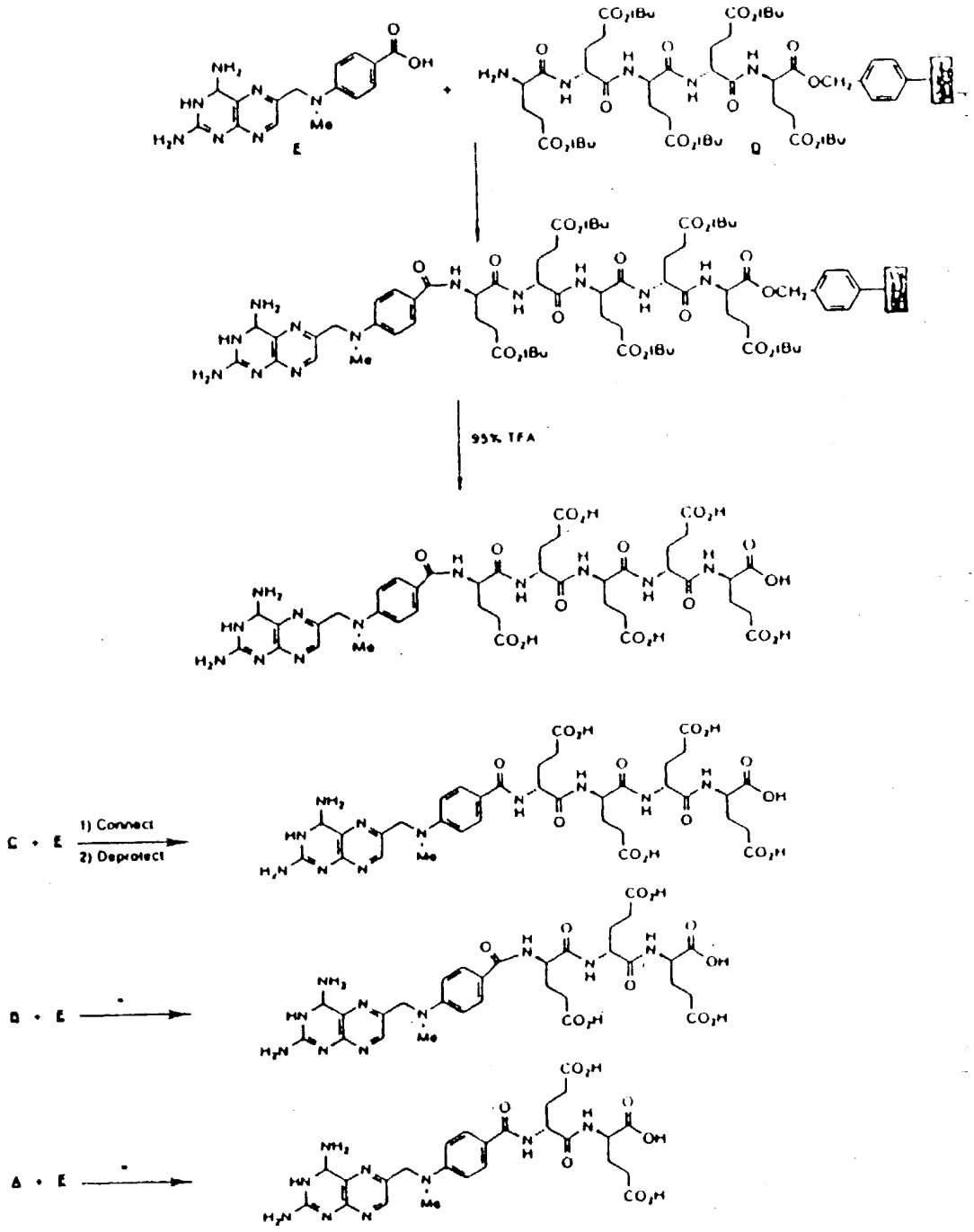


FIGURE 64

Sequence Analysis of microsatellite instability in PSM gene

Sample	Sequence	PSM EXPRESSION (IMMUNO STAIN)
Genomic	$T_9GC(TTTTG)_6(TTTG)_3T_7$	
LNCaP	$T_9GC(TTTTG)_6(TTTG)_3T_7$	positive
PC-3	$T_9GC(TTTTG)_8(TTTG)_3T_6$	negative
DU145	$T_{10}GC(TTTTG)_5(TTTG)_2T_7$	negative
T4 (tumor)	$T_{10}GC(TTTTG)_6(TTTG)_3T_7$	positive
N4(paired normal)	$T_9GC(TTTTG)_6(TTTG)_3T_7$	positive

FIGURE 65

Genomic Organization of PSM Gene

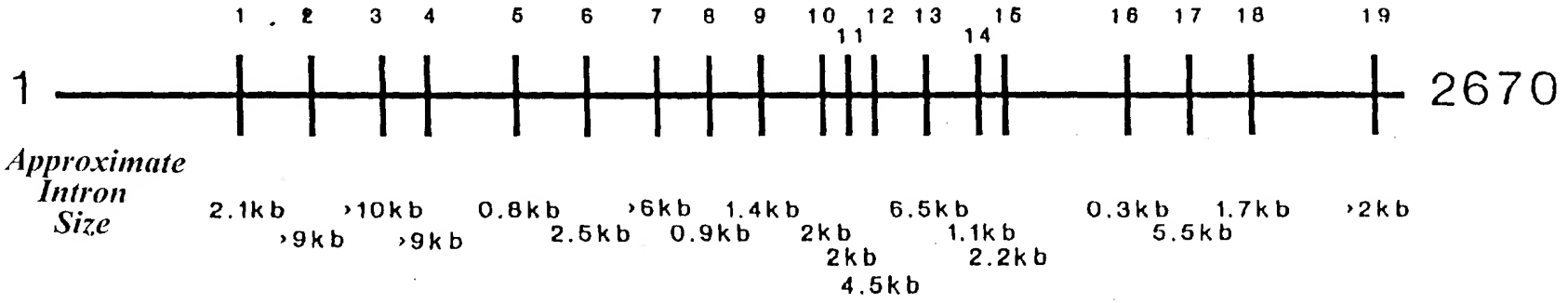


FIGURE 66

Location of microsatellite in PSM Gene